IMMOBILIZATION, CHARACTERIZATION AND USE OF A FISH PROTEASE

By

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Dedicated to:

My parents, who gave me the education and inculcated the virtues to reach high goals.

My husband, Dongming, who gave me the encouragement and motivation to complete this work.

FOREWORD

This thesis is presented in the form of original papers for publication in journals. The first two sections comprise of a general introduction presenting a comprehensive review of the literature on the topic as well as the rationale and objectives of the study. The next four sections constitute the main body of the thesis with each chapter representing a complete manuscript, and the last chapter is a summary of the major conclusions. The Faculty of Graduate Studies and Research, McGill University has approved this format in accordance with the conditions outlined in the Guidelines for Thesis Preparation, Thesis Specification, section 3 titled "Traditional and manuscript-based theses".

While the work reported in this thesis is the responsibility of the candidate, it was supervised by Dr. B.K. Simpson, Department of Food Science and Agricultural Chemistry, Macdonald Campus of McGill University.

ABSTRACT

Enzyme immobilization as a technique attaches free forms of enzyme molecules to stationary support materials to permit enzymes to be reused several times. Bovine trypsin as a model enzyme was immobilized onto controlled pore glass (CPG) beads to investigate the optimum conditions for immobilization, as well as the physico-chemical properties of the immobilized enzyme versus the free form of the enzyme. At pH 9, about 60% of the enzyme protein incubated with CPG was immobilized onto the CPG, and immobilized bovine trypsin activity was determined as 0.265 BAPNA U/g CPG beads. The immobilized bovine trypsin showed lower affinity for its substrate, lower susceptibility to inhibition by soybean trypsin inhibitor and higher thermal stability, while the optimum pH and optimum temperature values were shifted to higher values compared to those of the free enzyme. The immobilized enzyme was evaluated for its capacity to extract carotenoproteins from shrimp shell. After 11 re-uses, the immobilized enzyme retained about 77% of its initial activity, and the total yield of the product from the same immobilized trypsin was 4.3 times higher than a single use of the same amount of the free enzyme. Cunner fish is a cold water adapted, stomachless teleost fish. Cunner fish trypsin possesses some unique properties compared with homologous trypsins from (i) species acclimated to warm temperature regimes, and (ii) species with functionally distinct-stomachs. Cunner fish trypsin was

extracted from pancreatic tissue, and immobilized onto CPG beads using glutaraldehyde as cross-linking reagent. The influence of enzyme loading, the properties of the immobilized enzyme in terms of specific activities, and responses to pH and temperature were investigated. The kinetic properties and operational stability of the immobilized cunner trypsin were studied as well. The pH optimum of the immobilized fish trypsin shifted from pH 8.5 to pH 9, and the temperature optimum also increased from 45°C to 50°C versus the free form of the cunner enzyme. The catalytic efficiencies (V_{max} / K_m') of the immobilized fish trypsin were determined for both amidase and esterase reactions, using BAPNA and TAME as substrates and were found to be greater than those of immobilized bovine trypsin. Thus, the immobilized cunner fish trypsin had a higher catalytic capacity for the hydrolysis of both the amide and ester substrates. The operational stability of immobilized fish trypsin was studied by extracting carotenoprotein from shrimp shell. The immobilized fish trypsin retained 75% of its initial hydrolytic capacity after 11 re-uses, and the yield obtained was over 20% higher than that of immobilized bovine trypsin. When the immobilized cunner fish trypsin was applied to digest native pectin methylesterase (PME), it exhibited greater capacity to inactivate the PME than immobilized bovine trypsin. The inactivation efficiency of the immobilized fish trypsin was 20% higher than that of the immobilized bovine trypsin. The inactivation of PME was influenced by PME concentration, incubation time and temperature. In general, higher temperature, longer incubation period,

and lower initial PME concentration produced more PME inactivation. PME inactivated by immobilized fish trypsin and bovine trypsin regained part of its activity during storage at 4°C. The initial PME concentration affected the reactivation period. The kinetic studies indicated that the inactivation rate constants increased and *D*-values (time to inactivate 90% of the enzyme) decreased with increasing temperature for both immobilized fish trypsin and bovine trypsin. The activation energy (E_a) of PME inactivation by the immobilized fish trypsin was lower than that by the immobilized bovine trypsin, which explains why the immobilized fish trypsin had higher catalytic capacity at various temperatures than immobilized bovine trypsin.

RESUME

L'immobilisation d'enzymes est une technique consistant à immobiliser des molécules d'enzyme sous leur forme libre sur des matériaux stationnaires, afin de permettre la réutilisation de l'enzyme. La trypsine bovine, utilisée comme enzyme modèle, a été immobilisée sur des billes de verre à pores calibrés, dans le but d'étudier les conditions optimales pour l'immobilisation, ainsi que les caractéristiques physico-chimiques de l'enzyme immobilisée en comparaison à la forme libre. A pH 9, 60% environ de l'enzyme ont été immobilisés sur les billes et l'activité de la trypsine bovine immobilisée était de 0,265 BAPNA U/g billes. La trypsine bovine immobilisée a présenté une affinité plus faible pour son substrat, une sensibilité inférieure à l'inhibition par la trypsine de soja et une meilleure stabilité thermique, alors que le pH optimal et la température optimale étaient supérieurs à ceux de l'enzyme libre. La capacité de l'enzyme immobilisée à extraire les caroténoprotéines de la carapace de crevette a été évaluée. Après 11 utilisations, l'activité de l'enzyme immobilisée était de 77% de son activité initiale, et le rendement de production total était 4,3 fois plus important qu'avec l'utilisation unique de la même quantité d'enzyme libre. La tanche-tautogue est un téléostéen, vivant dans l'eau froide et ne possédant pas d'estomac. La trypsine de ce poisson possède quelques propriétés uniques par rapport à ses homologues (i) chez des espèces acclimatées à des climats chauds et (ii) chez des espèces polygastriques.

La trypsine de la tanche-tautogue a été extraite de tissus pancréatiques et immobilisée sur des billes de verre grâce à du glutaraldéhyde comme agent de couplage. L'influence de la charge enzymatique, les propriétés de l'enzyme immobilisée en terme d'activités spécifiques et la sensibilité au pH et à la température ont été étudiées. De même, les propriétés cinétiques et la stabilité opérationnelle de la trypsine immobilisée de la tanche-tautogue ont été analysées. Le pH optimal de l'enzyme est passé d'une valeur de 8,5 à 9 après immobilisation, et la température optimale a atteint la valeur de 50°C, alors qu'elle est de 45°C pour la forme libre. L'efficacité enzymatique (V_{max}/K_m) de la trypsine de poisson immobilisée a été déterminée pour les activités amidase et estérase, avec le BAPNA et le TAME comme substrats ; l'efficacité mesurée était supérieure à celle de la trypsine bovine immobilisée. Ainsi, la trypsine immobilisée de tanche-tautogue a présenté une capacité catalytique supérieure pour l'hydrolyse des fonctions amide et ester. La stabilité opérationnelle de la trypsine immobilisée de la tanche-tautogue après 11 utilisations était de 75% de sa capacité d'hydrolyse initiale et le rendement obtenu était de 20% plus important que celui de la trypsine bovine immobilisée. Lors de sa mise en présence avec de la pectine méthylestérase (PME) native, la trypsine immobilisée de tanche-tautogue a montré une plus grande capacité d'inactivation par digestion de la PME que la trypsine bovine immobilisée. L'efficacité d'inactivation de la trypsine de poisson immobilisée était de 20% plus importante que celle de l'enzyme bovine

immobilisée. L'inactivation de la PME était influencée par la concentration de la PME, par la durée d'incubation et par la température. En général, de températures plus élevées, des durées d'incubation plus longues et des concentrations initiales en PME plus faibles conduisent à une meilleure inactivation. L'inactivation de l'enzyme immobilisée bovine et de poisson recouvrent une partie de leur activité pendant leur stockage à 4°C. La concentration initiale en PME influençait la période de réactivation. Les études cinétiques ont indiqué que les constantes d'inactivation croissaient et que les *D*-values (temps d'inactivation de 90% de l'enzyme) décroissaient lorsque la température augmentait, pour les trypsines bovines et de poisson. L'énergie d'activation (E_a) de l'inactivation de la PME par la trypsine de poisson immobilisée était plus faible que pour la trypsine bovine immobilisée, ce qui explique pourquoi la trypsine de poisson immobilisée avait une meilleure capacité catalytique que la trypsine bovine immobilisée à différentes températures.

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Finally, my deepest gratitude goes to my husband Dongming for his love, patience, and support during the numerous trying moments.

CONTRIBUTION TO KNOWLEDGE

1. This is the first study on cunner fish trypsin immobilization and on immobilized fish trypsin enzyme characterization versus its immobilized mammalian counterpart.

2. The work also describes the first use of immobilized fish trypsin on inactivation of PME in fruit juices and the aid of the extraction of carotenoproteins from shrimp discards.

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LIST OF ABBREVIATIONS

aa-PEO	α-(2-aminoethylene amino)-ω-(2-aminoethylene amino)-poly(ethylene oxide)
ALR	Airlift reactor
AN	Acrylonitrile
ANOVA	Analysis of variance
APF	Ammonium persulfate
Asp	Aspartate
BA	Butyl acrylate
BAPNA	*N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide
Brij 35	Polyoxyethylene lauryl ether
BSA	Bovine serum albumin
CM-Sephadex	Carboxymethyl-Sephadex
CNBr	Cyanogen bromide
CPG	Controlled pore glass
CRL	Calculated risk level
CSTR	Continuous stirred tank reactor
DAO	Diamine oxidase
DEAE-cellulose	Diethylaminoethyl cellulose
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DTT	1,4-dithio-*DL*-threitol
DVB	Divinylbenzene
EA	Ethyl acrylate
EDTA	Ethylene-diamino-tetracetic acid
EF	Effectiveness factor

EGDMA	Ethylene glycol dimethacrylate
FBR	Fluidized bed reactor
Gln	Glutamine
HFCS	High fructose corn syrup
His	Histidine
HPMA	Hydroxypropyl methacrylate
ICI-IPAD	Ion chromatography with integrated pulsed amperometric detection
K_m'	Michaelis-Menten constant
LAAO	*L*-amino acid oxidase
L-GLDH	*L*-glutamate dehydrogenase
L-GLOD	*L*-glutamate oxidase
LPS	Lactoperoxidase
M.W.	Molecular weight
MSG	Monosodium glutamate
P(MMA-EA-AA)	Poly[(methyl methacrylate)-*co*-(ethyl acrylate)-*co*-(acrylic acid)]
PAGE	Polyacrylamide gel electrophoesis
PBR	Packed bed reactor
PELA	Poly-*DL*-lactide-co-poly-(ethyleneglycol)
PLGA	Poly-*DL*-lactide-colglycodide
PME	Pectin methyl esterase
PMMA	Poly(methyl methacrylate)
PPO	Polyphenol oxidase
PVCA	Poly vinylene carbonate
R_f	Retention factor
rpm	Revolutions per minute

SAPC	Succinamidopropyl-celite
SBTI	Soybean trypsin inhibitor
SDS	Sodium dodecyl
Ser	Serine
SPDP	*N*-succinimidyl-3-(2-pyridyldithio) propionate
TAME	*p*-toluene-sulfonyl-*L*-arginine methyl ester
TCA	Trichloroacetic acid
TEMED	*NNN'N'*-tetramethyl ethylene diamine
TMOS	Tetramethyl orthosilicate
Tris-HCl	Tris(hydroxymethyl) aminomethane hydrochloride
Tyr	Tyrosine
UV	Ultraviolet
V_{max}	Maximum reaction rate
τ	Tortuosity of support material

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CHAPTER I

GENERAL INTRODUCTION

Cunner fish (*Tautogolabrus adspersus*) is a cold water fish found in the waters of coastal region and offshore banks from Conception Bay, Newfoundland, Southwestern Gulf of St. Lawrence, south of New Jersey, and occasionally to the entrance of Chesapeake Bay (Coastal Ecology Group, 1989). The habitat temperature of cunner fish ranges from 4°C to 18°C; the species has been reported to tolerate seawater temperature slightly below 0°C, however, it is known to feed actively only when the temperature is ≥ 8°C (Steimle & Shaheen, 1999).

Digestive enzymes from cold water fish are a group of relatively less studied enzymes. However, they have been demonstrated to be more active catalysts at relatively low reaction temperatures compared with similar enzymes from mammals, thermophilic organisms and plant sources (Simpson & Haard, 1987). Because fishes are cold-blooded animals, their living habitat requires their enzyme systems to exhibit active forms at relatively low temperatures, such as 10°C (Kristjansson, 1991; Simpson & Haard, 1987). Cunner fish is peculiar cold water adapted fish in the sense that it lacks a well defined stomach (Chao, 1973). The stomach in higher vertebrates plays important functions in digestion. It secretes acid proteases to denature native protein molecules and partially hydrolyze these molecules. This pre-treatment of native protein molecules is very important for intestinal protease like trypsin and chymotrypsin to potentiate further

hydrolysis of proteins into simple peptides and amino acids (Simpson & Haard, 1987). Thus, without the help of acid proteases from the stomach, trypsin from stomachless species should be expected to be less efficient in digesting native proteins. However, Simpson and Haard (1987) reported that cunner fish trypsin had high initial rates and / or degree of hydrolysis for native protein molecules. Similar findings were made with stomachless species such as crawfish (Jeong *et al.,* 1994).

Harvesting and processing practices of the fishing industry is accompanied by co-production of large amounts of by-products / discards. About half of the fish weight is by-products, and most of them are currently discarded into the oceans as waste to pollute the environment (Gildberg, 1992). About 5% of the total fish processing discards mass is viscera material, which is a rich source of digestive enzymes (Castillo-Yanez *et al.*, 2005; Gildberg, 1992). Various digestive enzymes have been studied from fishes such as pepsin from Atlantic cod (*Gadus morhua;* Gildberg *et al.,* 1990), trypsin from rainbow trout (*Oncorhynchus mykiss*; Kristjansson, 1991), chymotrypsin from anchovy (*Engraulis japonica*; Heu *et al.*, 1995), collagenase from mackerel (*Scomber japonicus*; Park *et al.*, 2002), phospholipase A_2 from red sea bream (*Pagrus major*; Iijima *et al.,* 1997; Uchiyama & Iijima, 2005), *L*-glutamate dehydrogenase from Antarctic fish (*Chaenocephalus aceratus*; Ciardiello *et al.*, 2000).

Fish trypsins have been studied from various fish species, such as, starfish (*Dermasterias imbricate*; Camacho *et al.*, 1970), Greenland cod (*Gadus ogac*;

Simpson & Haard, 1984 a, b), eel (*Anguilla japonica*; Yoshinaka *et al.,* 1985), cunner (*Tautogolabrus adspersus*; Simpson & Haard, 1985, 1987), anchovy (*Engraulis encrasicholus*; Martinez *et al.,* 1988; Martinez & Serra, 1989), cod (*Gadus morhua L.*; Raae & Walther, 1989), rainbow trout (*Oncorhynchus mykiss*; Kristjansson, 1991), mullet (*Mugil spp*; Guizani, *et al.*, 1991; Pavlisko, *et al.*, 1999), anchovy (*Engraulis japonica*; Heu *et al.*, 1995), Antarctic fish (*Paranotothenia magellanica*; Genicot *et al.*, 1996), crawfish (*Procambarus clarkia*; Jeong *et al.*, 2000), chum salmon (*Oncorhynchus keta*; Sekizaki *et al.*, 2000), bigeye snapper (*Priacanthus macracanthus*; Benjakul *et al.,* 2003), lizard fish (*Saurida undosquamis*; Ohkubo *et al.,* 2004), Monterey sardine (*Sardinops sagax caerulea*; Castillo-Yanez *et al.*, 2005), Chinook salmon (*Oncorhynchus tshawytscha*; Kurtovic *et al.,* 2006), and yellowfin tuna (*Thunnus albacores*; Klomklao *et al.,* 2006).

Fish trypsins have essentially the same specificity as trypsins from mammalian and other sources (Castillo-Yanez *et al.,* 2005). Besides the traditional applications of mammalian trypsins, fish trypsins have specific applications due to their high catalytic activities at relatively low temperatures and higher capacity to hydrolyze native proteins (Simpson & Haard, 1987; Guizani *et al.,* 1991; Kurtovic *et al.*, 2006; Klomklao *et al.*, 2006). For example, certain food processing operations (*e.g.,* fermentation of fish products) require low reaction temperatures, which could minimize undesirable side reactions, and prevent the destruction of the heat-labile essential food components (Simpson & Haard, 1987; de Vecchi & Coppes, 1996; Simpson, 2000). The relatively low thermostabilities of fish

trypsins allow their ready inactivation under mild heat treatment after the desired processing (Simpson & Haard, 1987; Kristjansson, 1991; Simpson, 2000). Furthermore, their ability to denature native protein molecules may be beneficial in fruit juice processing for inactivation of undesirable endogenous enzymes such as polyphenol oxidases (PPO) in apple juice or pectin methylesterase (PME) in citrus juices, in order to improve the shelf life and quality of fruit juices (Simpson, 2000).

Trypsin purification is of particular interest to the food industry, because approximately 50% of the enzymes used as processing aids in food industry are proteases (Guizani *et al.*, 1991). These proteases are used to improve the handling characteristics, enhance the quality of food products, and extend the shelf life of food stuff, such as, in meat tenderization, cheese ripening, and dough conditioning / texture modification (Simpson, 2000).

The food industry requires more enzyme as food process aids for a number of reasons, *e.g.,* enzymes as bio-additives are perceived to be safe, more specific to substrates, and more acceptable to the customers compared with chemical additives (Taylor & Leach, 1995). Thus the exploration of new sources of useful enzymes for the growing enzyme market is receiving more attention by researchers (Yoshinaka *et al.,* 1985; Roy *et al.*, 1996; Sekizaki *et al.*, 2000; Kurtovic *et al.*, 2006). Discovery of new sources of enzymes from fishing industry by-products not only provides new uses of the "waste" but also makes more profits for the fishing industry (Kurtovic *et al.*, 2006).

However, extraction of enzymes from fish for commercial use is often not practical for various reasons. The extraction procedures are time consuming and result in low yields. The activity of final product is subject to variations in the source materials and operation conditions. Enzyme activities are different from batch to batch. Moreover, the raw material is subject to seasonal variations and political and environmental restrictions that regulate the harvesting of fish species (Simpson, 2000)

Enzyme immobilization could make the enzymes reusable, thereby reducing processing cost (Salis *et al.,* 2005; Ikeda & Kurokawa, 2002). Immobilization could also enhance the thermal and handling stabilities of the enzyme (Vidinha *et al.*, 2006). Because the immobilized enzymes can be removed from the material or products easily, it may provide better quality control of the final products compared with that of the free form (Gomez *et al.*, 2006; Giacomini *et al.*, 2001). It is also possible to immobilize a few enzymes to the same support at the same time to permit the use of multiple enzyme system in food processing (Basu *et al.*, 2005).

Bovine trypsin immobilization has been widely investigated by many researchers (Sear & Clark, 1993; Ge *et al.*, 1996 b; Chase & Yang, 1998; Isgrove *et al.*, 2001; Wilcox *et al.*, 2002; Migneault *et al.*, 2004; Kang *et al.*, 2005; Wu *et al.*, 2005). Both natural and synthetic polymers have been investigated as support materials. And research is still ongoing to discover better support materials or immobilization methods, in order to facilitate applications (Huang *et al.,* 2006).

However, few studies on fish trypsin immobilization were found in the literatures (Amaral *et al.*, 2006).

Certain enzymes from cold water fish (*e.g.*, cunner trypsin) are known to display subtle differences from their counterparts from mammals (*e.g.*, bovine trypsin), such as different pH, temperature, and kinetic properties (Simpson & Haard, 1987). Based on the differences in characteristics between the free forms of bovine versus fish trypsins, it is speculated that immobilized bovine and fish trypsins may also exhibit important differences to make them more or less suited for particular applications. Therefore, studies on fish trypsin immobilization are necessary to derive the basic knowledge needed to rationalize or justify their use as processing aids.

In this study, bovine trypsin immobilization was investigated as a model for subsequent studies with cunner trypsin. The conditions of immobilization, some properties of the immobilized fish trypsin and applications of the immobilized fish trypsin were studied as the subject of this thesis. This research could facilitate the application of the cunner fish enzyme in commercial processes.

CAPTER II

LITERATURE REVIEW:

ENZYME IMMOBILIZATION

2.1 Introduction

The use of enzymes is well established and widespread in the food industry. Enzymes as catalysts have many advantages, such as reducing processing cost, increasing yields of extracts from raw materials, improving handling of material and improving the shelf life and sensory characteristics of food. The main advantages in using enzymes instead of chemical treatments are that enzymatic reactions are (i) carried out under mild conditions (such as, pH, temperature and pressure), and (ii) are highly specific, thus reducing the occurrence of undesirable side reactions and by-products in the final product (Taylor & Leach, 1995; Law, 2002).

Enzyme immobilization is a technique specially designed to attach enzyme molecules to solid supports over which the substrate is passed and converted to products. By using this technique, the fixed or bound enzyme molecules can be recovered and reused over and over again (Bickerstaff, 1997a; Brena *et al.*, 2003).

Enzyme immobilization has been investigated and used for many years. Immobilized enzymes show distinct characteristics compared with the free forms of the enzyme (Lee & Swaisgood, 1997; Clark & Bailey, 1983; Brena *et al.*, 2003; Nahalka & Gemeiner, 2006). The immobilized enzyme can be packed into a column, so that the soluble substrate can be passed through the column, for the product to be produced and collected at the end of the column. Hence, a continuous enzymatic operation can be used in industry, rather than the batch to batch approach using the soluble or free form of the enzyme. After the enzymatic reaction, the immobilized enzyme can be easily removed from the product, thus, the quality of final product can be controlled easily, and contamination due to the added enzymes can be minimized. Several enzymes can be immobilized to the same support material, so that multiple enzyme systems can be achieved easier than that of the free forms (Basu *et al.,* 2005; Sutherlin & Rodwell, 2004; Limoges *et al.,* 2006). The immobilization technique also can increase the stability of the enzymes, thus the enzyme can be used over wider range compared with its free counterpart (Boadi & Neufeld, 2001; Colletier *et al.*, 2002, Zhu *et al.*, 2005a).

Nevertheless, there are some drawbacks with enzyme immobilization, such as the cost of immobilization, loss of enzyme activity during immobilization, greater initial plant investment, more technically complex process requirement for more skilled personnel or supervision, unique sanitation and toxicology problems, and applicability of the technique mainly to soluble substrates (Nahalka & Gemeiner, 2006; Brena *et al.,* 1998).

Taking into account the intended use and application, the first consideration is to decide on the support material and the method of immobilization. Some points to be considered when taking a decision are listed in Table 2.1 (Bickerstaff, 1997a). It involves the physical and chemical properties of the support material, stability of the immobilized enzyme, safety of the immobilized enzyme used in food, pharmaceutical and medical applications, economic profiles for preparation and application of immobilized enzyme (Bickerstaff, 1997a).

2.2 Methods used in enzyme immobilization

Mainly, there are five chemical and physical methods used in enzyme immobilization. These are covalent binding, adsorption, entrapment, encapsulation, and cross-linking (Fig. 2.1). Chemical methods involve the formation of covalent bonds between residues of an enzyme and a functional, water-insoluble polymer (covalent binding), or between two or more enzyme molecules (cross-linking). Adsorption, entrapment and encapsulation are physical phenomena and involve electrostatic interactions, formation of ionic bonds and protein-protein interactions, to form the connection between the enzyme molecules and the support material. Cross-linking also can be achieved by physical methods, *e.g.,* flocculation (Bickerstaff, 1997a).

Among these methods, covalent binding is one of the popular methods. It has been studied by many researchers, and versatile kinds of supports (*e.g.*, controlled pore glass beads, agarose, chitin and chitosan) have been applied with

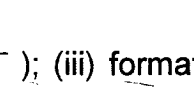
covalent binding (Tiller *et al.,* 1999; Kim *et al.*, 2001; Soares *et al.,* 2001; Kartal & Kilinc, 2006). Other methods (adsorption, encapsulation, entrapment, cross-linking), have been carried out by different investigators (Dautzenberg *et al.*, 1997; Huang *et al.,* 2005; Jan van unen *et al.,* 2001; Castellanos *et al.*, 2002; Kilinc *et al.*, 2006). All these methods can be used by themselves; also they have been used in combination with each other to enhance the properties of the immobilized enzyme.

2.2.1 Covalent binding

Covalent binding method is a very effective method (Zhou *et al.,* 2001). It is easy to carry out – enzyme molecules are added to a suspension of the support and any non-covalently bound enzyme is removed by suitable washings. The covalent bond is formed between the functional groups of the supports and enzyme (Fig. 2.1). The functional groups belonging to the enzyme involve amino groups (NH_2) of lysine and arginine, the carboxyl groups (CO_2H) of aspartic acid and glutamic acid, the hydroxyl groups (OH) of serine and threonine, and the sulfydryl group (SH) of cysteine (Bickerstaff, 1997a).

Coupling an enzyme molecule and support material could be achieved by several methods. However, most reactions involve the following covalent bond formation:

(i) formation of an isourea linkage ($-NH-\overset{\overset{O}{\|}}{C}-NH-$); (ii) formation of a diazo

linkage (); (iii) formation of peptide bonds (); and an (iv) alkylation reaction (Cen *et al.*, 2003; Bickerstaff, 1997a).

The choice of which reaction to use depends on the functional groups of the active sites in the enzyme molecules. The covalent bond should not involve the critical amino acid residues of the active site of the enzyme, which could cause inactivation of the enzyme (Bickerstaff, 1997a). For example, if the active site of the enzyme involves a carboxyl group, it is better to choose a method based on reaction to amino groups to form the covalent bonds rather than carboxyl groups.

The covalent binding of an enzyme to a support material offers numerous advantages as a method for enzyme immobilization (Malmsten *et al.,* 1999; Malmsten & Larsson, 2000; Cardoso *et al.,* 2005). The coupling between enzyme and a functional support is easy to carry out. Enzyme and polymer suspension are mixed together to allow the coupling to proceed, and any noncovalently bound enzyme could be removed by suitable washings. The immobilized catalyst can be quickly removed from reaction system, thus it is easy to control the stage of the enzymatic reaction and selective or particular products could be achieved. Because of the variations in the chemical and physical nature of the support, these immobilized enzymes can be adapted to a variety of specific engineering requirements (Malmsten & Larsson, 2000). A most distinguishing feature of this method for immobilizing enzymes is that it is a chemical method, a derivatized enzyme is produced, which may exhibit superior chemical or physical properties

relative to its soluble counterpart (Limoges *et al.,* 2006; Yinghui *et al.,* 2002; Sahin *et al.,* 2005).

Of course, there are certainly some disadvantages to this method of enzyme immobilization. The successful attachment of an enzyme to a support requires elaborate preparations of both the enzyme and the support. This chemical method can give derivatives of unsuitable physical character, can exhibit low efficiency in the coupling procedure, and can give derivatives of low catalytic efficiency (Bickerstaff, 1997a; Boadi & Nenfeld, 2001; Ramakrishnan *et al.,* 2006).

There are various supports used in covalent binding (Table 2.2). Different supports include different functional groups to form the covalent bond with the enzyme molecules. On the other hand, chemical modification may produce derivatives containing different functional groups by introducing some normal functional groups onto the supports, in order to cause stronger connection with the enzyme molecules. Thus, supports and their derivatives make this method more feasible (Cen *et al.*, 2003).

2.2.1.1 Some supports used in covalent binding

2.2.1.1.1 Controlled-Pore Glass (CPG)

Many studies have been carried out on enzyme immobilization onto CPG (Clare *et al.,* 2001). CPG is produced from a borosilicate base material, which is heated to separate the borates and the silicates. The borates are leached out from the

material, leaving the silica glass with uniform, controlled pores (Ghosh & Musso, 1987).

CPG is an improvement over traditional soft gels, especially in processes that require fast flow rates or high pressures because the flow rates with CPG are linear with pressure (Limbut *et al.,* 2004). CPG is a glass structure and is immune to biological degradation (Morais *et al.,* 1997). This matrix is compatible with almost all organic solvents and concentrated acids (except hydrofluoric acid) (Rosa *et al.,* 2002). As a solid support, CPG eliminates many of the problems experienced with gels. For example, CPG has a high surface area offering ligand coupling efficiency and high yields. The narrow pore size distribution also provides high yields with high purity. The mechanical strength of CPG provides reproducible results with constant column parameters. In addition, CPG is thermostable and autoclavable making its use in a sterilized environment a reality (Rosa *et al.,* 2002; Maquieira *et al.,* 1994).

Enzymes such as sulfhydryl oxidase (Janolino & Swaisgood, 1982), trypsin (Taylor & Swaisgood, 1980; Chen *et al.*, 1994; Clare *et al.,* 2001; Sears & Clark, 1993), urease (Limbut *et al.,* 2004), beta-glycosidase (Petzelbauer *et al.,* 2002), nitrite reductase (Rosa, 2002) were immobilized onto the CPG successfully. Various agents may be used to activate CPG, *e.g.,* cyanogen bromide, gularaldehyde, succinic anhydride (Bickerstaff, 1997a; Janolino & Swaisgood, 1997). However, glutaraldehyde-CPG and succinylated-CPG are the preferred ones (Janolino & Swaisgood, 1982; Sears & Clark, 1993; Petzelbauer *et al.,* 2002;

Kartal & Kilinc, 2006). Both of them have been used by different researchers, and the results showed that enzymes could be bound to the support effectively and showed more stable properties compared with the free enzymes (Kartal & Kilinc, 2006).

2.2.1.1.2 Polysaccharides

Polysaccharide polymers are popular support materials for enzyme immobilization (Chen *et al,* 1997). For example, cellulose, dextran (Sephadex), starch, agarose (Sepharose), chitin and chitosan have been used in enzyme immobilization (Liu *et al,* 1997; Pereira *et al.,* 2005; Mansee *et al.,* 2005). The sugar residues in these polymers contain hydroxyl groups for chemical activation for covalent bond formation (Martin *et al.,* 1998). Also, hydroxyl groups form hydrogen bonds with water molecules and thereby create an aqueous (hydrophilic) environment in the support material. However, the polysaccharide supports are susceptible to microbial/fungal disintegration, and organic solvents can cause shrinkage of the gels (Martin *et al.,* 1998).

2.2.1.1.2.1 Dextran and Agarose

Agarose and dextran could be used as supports by themselves, also could be derivatised to other forms (Fermi *et al.,* 1998). Sepharose® is the registered trademark for spherical agarose gel particles produced by Pharmacia Fine Chemicals, Inc. Agarose is a linear polysaccharide consisting of alternating

residues of D-galactose and 3,6-anhydro-*D*-galactose units. Similarly, Sephadex® is the registered trademark for spherical dextran gel particles produced by Pharmacia Fine Chemicals, Inc. Sephadex is a modified dextran whose molecules are crosslinked to produce networks of varying degrees. To achieve immobilization, Sephadex is activated by cyanogen bromide (chloride or iodide), glutaraldehyde and thiolsulfinate (Giacomini *et al.,* 2001). The cyanogen-bromide-activated Sephadex has a reactive imidocarbonate which subsequently reacts with the enzyme (Gabel, 1973). Glutaraldehyde-gel is based on the reaction of the protein amino groups with aldehyde moieties, and the thiolsulfinate-gel is based on the reaction between the protein's thiol groups with disulfide oxide reactive structures on the agarose gel (Guisan *et al.,* 1997; Batista-Viera *et al.*, 1994). The glutaraldehyde-based chemistry is a very traditional and effective method for immobilization and stabilization. The second method is interesting because even though a covalent bond is formed between enzyme and the matrix, this bond is of disulfide type and can be broken by reaction with reducing agents such as 1,4-dithio-*DL*-threitol (DTT) (Guisan *et al.,* 1997). This fact makes it possible to elute the enzyme when the activity has decayed after use, regenerate the reactive groups on the support, and re-load it with fresh enzyme (Guisan *et al.,* 1997). This technique was used to immobilize trypsin by Gabel (1973). The results showed that the enzyme is stable towards denaturation by urea.

2.2.1.1.2.2 Chitin and Chitosan

The polysaccharides that have been used for enzyme immobilization include chitin and chitosan. Chitin is a natural polymer with a structure similar to cellulose, i.e., *N*-acetyl-*D*-glucosamine and glucosamine are copolymerised by β-1,4 linkage. It has a molecular weight of 2-3 million daltons. Like cellulose, chitin is insoluble in water but can be converted to water-soluble derivatives, much in the same way as cellulose is converted to useful water-soluble derivatives (Magalhaes & Machado, 1993).

Chitin is found in the tough, fibrous exoskeletons of insects, crustacea, and in some fungi. Removal of *N*-acetyl groups from chitin to produce chitosan takes place by deacetylation with concentrated alkali. During the deacetylation reaction, some alkaline cleavage of the polysaccharide molecule occurs, resulting in a decrease in polymer size and viscosity. Chitosan is a soluble biopolymer or polysaccharide at pH values less than 7, but preferably below pH 5, and most often in solutions of dilute organic acids (0.1%). The soluble chitosans usually contain above 70% *D*-glucosamine (Gemeiner *et al.*, 1982).

Chitin possesses excellent adsorption capacity due to its high porosity and elasticity (Yoksan *et al.*, 2003). In addition, it provides the active sites for chemisorption owing to the existence of functional hydroxyl and amine groups (Shi *et al.*, 2006). It is a highly charged anionic polyelectrolyte. The conversion of chitin to chitosan is accompanied by exposure of more amino groups and exhibits high adsorptive activity (Zeng & Ruckenstein, 1998). Therefore, chitin and

chitosan are used in ion exchange resins, coagulants, medicines and as carriers for immobilization of enzymes (Shi *et al.,* 2006; Gomez *et al.*, 2006).

As supports, chitin and chitosan are considered to have high potential because of certain remarkable characteristics including the facile immobilization either by chemical reaction or physical adsorption, low bulk density, coarse porous structure, and non toxicity (Guo *et al.,* 2003). Chitosan may be used more conveniently than chitin owing to the presence of more amino ($-NH_2$) groups on chitosan chains that serve as the coordination and reaction sites. In addition, chitosan is well known as an ideal support for enzyme immobilization because of its many features such as hydrophilicity, biocompatibility, biodegradability, and anti-bacterial properties (Guo *et al.,* 2003; Li & Hsieh, 2006).

Immobilization of various enzymes onto chitin and chitosan has been reported (Ge & Zhang, 1993; 1996a; 1996b; Huang, 1989; Machiunska *et al.*, 2000; Vaillant *et al.,* 2000). Usually, it is by using chemical modification to enhance the capability of holding enzymes. Some advantages with chemical modification are that it can (i) prevent chitosan solids from dissolution in comparatively strong acid solutions (for example, pH less than 2); (ii) improve mechanical strength and resistance to chemical degradation; (iii) increase porosity and improve superficial areas of the beads; and (iv) improve diffusion properties and accessibility to internal sites (Rorrer *et al.,* 1993).

In most cases, glutaraldehyde is used to hold the enzymes. For example, β-galactosidase (Machiunska *et al.,* 2000) and glucoamylase (Stanlet *et al.,* 1978) immobilized onto chitin with glutaraldehyde exhibited as high as 60-70% of the activities of the native enzymes. Acid phosphatase was immobilized on the chitosan beads by Juang *et al.,* (2001). The acid phosphatase immobilized onto the cross linked beads retained about 80% of the original activity, and the lifetime of immobilized enzyme was extended up to 42 days in this case.

However, glutaraldehyde can make the chitin matrix brittle and induce brown pigment formation on the immobilized enzyme (Ge *et al.,* 1996b). As a result, the specific activity and activity recovery of the immobilized enzyme were very low. Furthermore, it was impossible to regenerate the unbound enzyme for reuse in enzyme immobilization. To overcome this drawback, they used formaldehyde as an ideal substitute for glutaraldehyde. Because the formaldehyde molecule is much smaller than glutaraldehyde, unwanted cross-linking was prevented both inside the enzyme and between the enzyme molecules. Also formaldehyde has a good safety record in food industrial applications. From their research, trypsin immobilization with formaldehyde was much better than the method with glutaraldehyde. The specific activity, activity recovery, storage/operational stability were enhanced. And the recovered unbound enzyme was reusable for enzyme immobilization (Ge *et al.,* 1996b).

Kurita *et al.,* (1997), also reported that the glutaraldehyde method had problems with the remaining aldehyde groups and possible elution of the aldehyde as a

result of hydrolysis during operation. They introduced the mercapto groups to form mercapto-chitin as supports, since mercapto groups readily form disulfide linkages with the mercapto or disulfide groups of enzymes. In this study, acid phosphatase was immobilized onto two kinds of mercapto-chitins: 2-mercapto- and 6-mercapto-chitins. The 6-mercapto-chitin/enzyme conjugate retained high activity even after repeated uses in batch systems.

2.2.1.1.3 Synthetic Polymers

Special attention has been paid to synthetic supports because (i) synthetic matrices are resistant to pH, temperature, and biological degradation (Petro *et al.*, 1995; Wu *et al.*, 2005); (ii) their hydrophobic-hydrophilic properties can be easily altered by appropriate selection of co-monomers used during the synthetic step (Chase *et al.*, 1998); (iii) their morphology might be altered by selection of type and amounts of inert diluents in the polymerization step; and (iv) surface modifications are easily achieved and various reactive groups may be anchored onto the matrix (Nouaimi *et al.*, 2001; Basinska *et al.*, 2005). Moreover, the concentration of these groups may be controlled by changing the extent of modification (Ge & Zhang, 1993; Petro *et al.*, 1995; Bryjak & Kolarz, 1998; Nouaimi *et al.*, 2001; Li *et al.*, 2001; Wu *et al.*, 2005).

Vinyl polymers, as a class, have been widely explored as supports for enzyme immobilization since by proper choice of monomers and polymerization conditions, almost any desired combination of mechanical and chemical

properties can be attained in principle in the final product (Chase *et al.,* 1998; Ding *et al.,* 2001; Allard *et al.*, 2002).

Polystyrene was the first synthetic polymer to be used for the immobilization of antibodies, antigens, and enzymes (Goldstein & Manecke, 1976). Nowadays, there has been some renewed interest in polystyrene as support mainly because of its availability and low price. Polystyrene anion-exchange resin was used as matrix for trypsin immobilization (Ge & Zhang, 1993; Urek & Pazarlioglu, 2004).

Acrylic copolymer is another big group of synthetic supports. Based on the shortage of detailed studies concerning the effect of synthetic carriers on enzyme immobilization, Bryjak *et al.,* (1998), investigated covalent binding of trypsin onto various acrylic carriers. Acrylic copolymers cross-linked with various amounts of the cross-linking agents such as divinylbenzene (DVB), ethylene glycol dimethacrylate (EGDMA), or trimethylolpropane triacrylate (TMPA) were synthesized by suspension polymerization. Acrylonitrile (AN), butyl acrylate (BA), ethyl acrylate (EA) and hydroxypropyl methacrylate (HPMA) were used as co-monomers. The result showed that the support's superstructure depends mainly on the kind of monomers, solvatation conditions during polymerization, cross-linking ratio, and amount of anchor groups (Bryjak *et al.,* 1998). Modification of copolymers obtained by aminolysis with ethylenediamine occurs mainly on porous surfaces. High level of modification can cause a critical alteration of carrier morphology. Trypsin was immobilized on such carriers. A comparison of the properties of the immobilized enzymes and native trypsin demonstrated that

the binding of the protein to the carrier caused an increase of the storage and pH-stability of the bound enzyme (Bryjak *et al.,* 1998).

Furthermore, the solubility of synthetic polymers can easily be made reversible by changing the physical conditions, such as pH, temperature and addition of certain ions (Alonso-Morales, 2004; Guoqiang *et al.,* 1995). By these properties, we could overcome the drawback of the insoluble carriers in bioreactor with slow binding/catalysis ability, clogging of the pores due to diffusion-controlled mass transfer, steric hindrance (Nahalka & Gemeiner, 2006). Thus, if enzymes are immobilized onto such carriers, they can be used for biocatalysis in soluble form and recovered by precipitation for reuse (Zhu *et al.,* 2005b; Nahalka & Gemeiner, 2006). The copolymer of methacrylic acid and methyl methacrylate possesses such properties, and it can be made soluble-insoluble by changing pH or addition of calcium ions with ethanol or temperature change (Guoqiang *et al.,* 1995). Covalent binding of enzymes to reversibly soluble polymers requires accurate choices and development of the proper washing protocol to eliminate noncovalently bound protein (Arasaratnam *et al.,* 2000; Alonso-Morales, 2004). Trypsin was immobilized onto such carriers by covalent binding (Arasaratnam *et al.,* 2000). When optimized conditions were used for coupling, about 90% of trypsin was covalently attached to the matrix and maintained 100% activity after three cycles of precipitation-dissolution (Arasaratnam *et al.,* 2000). Soluble enzyme-polymer conjugates showed higher affinity toward the lower molecular weight substrate as observed for the free enzyme. But the activity of the conjugate toward high-molecular weight substrates decreased compared to low-

molecular weight substrates. This could be a limiting factor (Arasaratnam *et al.*, 2000).

The synthetic polymers also could be used by introducing some functional groups to enhance the properties of the supports. For instance, copolymers of poly (vinylene caronate) and α-(2-aminoethylene amino)-ω-(2- aminoethylene amino)-poly (ethylene oxide) were prepared by Ding *et al.*, (2001). It is well known that cyclic carbonate groups can readily combine with amino groups of biomolecules under very mild conditions (Ding *et al.*, 1999; Huo *et al.*, 2004). Moreover, these highly reactive groups show good stability under standard conditions, *e.g.*, stored in aqueous solution at a neutral pH (Ding *et al.*, 1999). Generally cyclic carbonate groups can be introduced onto the polymer supports by treatment of polymers containing hydroxyl groups with ethyl chloroformate in an anhydride organic solvent or even directly by the copolymerization of vinylene carbonate with some hydrophilic vinyl monomers (Ding *et al.*, 2001). Chains of poly vinylene carbonate (PVCA) contain cyclic carbonate groups as many of its structural units. The cyclic carbonate groups also can react with amino groups under very mild reaction conditions and has thus been employed as effective functional groups for enzyme immobilization. However, due to its great hydrophobicity, PVCA itself cannot directly be used as support material for enzymes (Huo *et al.*, 2004; Ding *et al.*, 2001; Doretti *et al.*, 1999). It is known that hydrophobic materials may cause inactivation of enzymes as organic solvents may do. In addition, supports swollen in aqueous solution will favor the diffusion of enzyme molecules into the supports and provide a comfortable microenvironment for enzymes. With those concern in

mind, α-(2-aminoethylene amino)-ω-(2-aminoethylene amino)-poly(ethylene oxide) (aa-PEO) was produced by crosslinking reaction between PVCA and aa-PEO (Ding *et al.,* 2001). As the crosslinking reaction between PVCA and aa-PEO was completed, cyclic carbonate groups remaining in the supports could be employed as the functional groups for enzyme immobilization. Trypsin was immobilized onto such a support by covalent binding. The specific activity of immobilized trypsin and amount of enzymes coupled to supports were related to the proportion of aa-PEO and PVCA, reaction time, enzyme concentration and pH value of enzyme solution (Ding *et al.,* 2001). The amount of trypsin coupled and specific activity reached their maximum at PVCA / aa-PEO = 1:2. The optimal pH for immobilized trypsin shifted to high values compared with the optimal pH for the free trypsin. The K_m' of the immobilized trypsin was higher than that of free trypsin (*e.g.,* 32.5 mmol/L and 15.2 mmol/L for immobilized and free trypsin, respectively) (Ding *et al.,* 2001).

2.2.2 Adsorption

Enzyme immobilization by adsorption is the simplest method and involves reversible surface interactions between enzyme and support material (Fig. 2.1) (He *et al.,* 2006; Biegunski *et al.,* 2005). The forces involved are mostly electrostatic, such as van der Waals forces, ionic and hydrogen bonding interactions, although hydrophobic bonding can be significant. These forces are very weak, but sufficiently large in number to enable reasonable binding (Bickerstaff, 1997a). Existing surface chemistry between the enzyme and support

is utilized so no chemical activation/modification is required and little damage is normally done to the enzymes by this method of immobilization (Torres *et al.*, 2004). The procedure consists of mixing together the biological components and support with adsorption properties, under suitable conditions of pH, ionic strength, and temperature, for a period of incubation, followed by collection of the immobilized material and extensive washing to remove nonbound biological components (Yan *et al.*, 2002).

Because there is little or no damage to enzymes, the enzymes could keep their activities as much as possible compared to its free form. However, because of the forces joining the enzyme and support are weak, and easily affected by changing pH or ionic strength conditions, leakage of enzymes from the support is the main disadvantage of this method (Lee & Swaisgood, 1997; Vidinha *et al.*, 2006). Desorption can occur under many conditions; for instance, changes in pH, temperature, and ionic strength will promote desorption (Lee & Swaisgood, 1997; Trivedi *et al.*, 2005). To overcome the leakage, Tyagi and co-workers (1994) modified the enzyme molecules (trypsin) with pyromellitic dianhydride (PMDA) to increase its surface negative charges, and then immobilized the modified trypsin onto DEAE-cellulose, in order to enhance the strength of binding. By using this method, it is also possible to control the strength of binding by varying the extent of chemical modification.

Nonspecific binding can become a problem with the adsorption approach, if the substrates, products, and/or residual contaminants are charged and interact with

the support (Malmsten *et al.,* 1996; Isgrove *et al.,* 2001). This can lead to diffusion limitations and reaction kinetics problems, with consequent alteration in the kinetic parameters V_{max} and K_m' (Fuentes *et al.,* 2006; Liu *et al.,* 2001). Further, binding of protons to the support material can result in an altered pH microenvironment around the support with consequent shift in pH optimum (1-2 pH units), which may be important for enzymes with precise pH optimum requirements (Mustranta *et al.,* 1993; Wang *et al.,* 2006a). Unless carefully controlled, overloading the support can lead to low catalytic activity, and the absence of a suitable spacer between the enzyme molecule and the support can produce problems related to steric hindrance. Non-specific binding could be overcome by using some compounds (*e.g.,* casein and amino acid) to block the non-specific site before immobilization, and then removing these compounds after immobilization (Isgrove *et al.*, 2001).

Adsorption is one of widely used methods for enzyme immobilization (Dautzenberg *et al.,* 1997; Huang *et al.,* 2005; Numata *et al.,* 2006; Salis *et al.,* 2005; Liu *et al.,* 2006). Several enzymes (*e.g.,* beta-glactosidase, lipase, invertase) have been immobilized onto different supports (*e.g.,* bone powder, celite, poly(acrylamide/maleic acid) hydrogels) by means of adsorption (Carpio *et al.* 2000; Khare & Nakajima, 2000; Arslan *et al.,* 2000; Fernandez-Lorente *et al.*, 2001). Immobilization of enzymes by adsorption onto inorganic supports has been proposed for its simplicity, low cost, as well as preservation of the enzyme/substrate specificity (Carpio *et al.,* 2000; Khare & Nakajima, 2000). Silica gel is among the most used inorganic supports and consists of an amorphous

inorganic polymer having silicane groups (Si-O-Si) on the inside and silanol groups (Si-OH) on its surface; these silanol groups react chemically with distinct ionic species and create different surface properties depending on pH (Prado *et al.,* 2004). Silica gel also possesses a well-established particle size, a well-defined porosity, a high surface area and a high mechanical, chemical and thermal stability, as well as low cost which makes it an ideal support for immobilization (Branyik *et al.,* 2000). The effects of temperature on the storage stability of chlorophyllase activity, the effects of different incubation temperatures for long-term reaction processes, and the effects of re-cycling on the enzyme activity of the immobilized chlorophyllase absorbed onto silica gel were studied (Kermasha, 2000). The result showed that the immobilized chlorophyllase could keep its highest activity at 30°C for 6 h incubation and could be reused for 5 cycles. In contrast, the free enzyme lost 60% activity after 3 h incubation. Limit dextrinase partially purified from rice seeds was immobilized by adsorption on γ-alumina beads (Furegon *et al.,* 1997). Binding and activity yields were 88% and 52%, respectively. The adsorbed enzyme showed a broader pH optimum and an increased thermal stability compared to the free one.

2.2.3 Entrapment

Immobilization by entrapment differs from adsorption and covalent binding in that enzyme molecules are free in solution, but restricted in movement by the lattice structure of a gel (Fig. 2.1) (Bickerstaff, 1997a). The porosity of the gel lattice is controlled to ensure that the structure is tight enough to prevent leakage of the

enzyme molecules, yet at the same time allow free movement of substrates and products. Inevitably, the support will act as a barrier to mass transfer (Bickerstaff, 1997a).

There are several major methods of entrapment: (1) ionotropic gelation of macromolecules with multivalent cations (*e.g.,* alginate) (Zhu *et al.,* 2005a; Srivastava *et al.,* 2005); (2) temperature-induced gelation (*e.g.,* agarose, gelatin) (Betigeri & Neau, 2002; Schuleit & Luisi, 2001); (3) organic polymerization by chemical / photochemical reaction (*e.g.,* polyacrylamide) (Mersal & Bilitewski, 2005; Ziomek *et al.,* 1984); (4) precipitation from an immiscible solvent (*e.g.,* polystyrene) (Volodkin *et al.,* 2003).

Entrapment of enzyme within the natural biopolymers attracts more interests for the researchers because of their safety. Tembe and co-workers (2006) introduced polysaccharide – polysaccharide binding to entrap enzyme. They used agarose and guar gum, two natural biopolymers as composite materials, because they are highly permeable toward water, good adhesive and non-toxic. These two biocompatible polymers could overcome the brittleness of pure guar gum matrix and its shrinkage. κ-carrageenan also was introduced to improve the stability of gel (Tembe *et al.,* 2006). Sahin *et al.,* (2005), reported a novel matrix based on alginate and κ-carrageenan. Such matrix showed advantages like high immobilization capacity and good mechanical stability for various biotechnological applications such as a part of enzyme electrode or enzyme reactor (Sahin *et al.,* 2005).

Sol-gel materials are another class of materials used to form lattice structure for entrapment enzyme (Jan van unen *et al.,* 2001). Sol-gel materials for enzyme entrapment are made from tetramethyl orthosilicate (TMOS) as the silicon precursor (Ei-Rassy *et al.,* 2003). Acetylcholinesterase was immobilized in a sol-gel and used as a biosensor for the determination of acetylcholine (Doong & Tsai, 2001). The results showed a good linearity of acetylcholine in the range from 0.5 mM to 20 mM (R^2=0.98) by this biosensor, which indicated the developed biosensor is suitable for the analysis of acetylcholine. But the extension of this technique has been limited by two shortcomings of sol-gel materials: their brittleness and narrow pore network (Shen & Tu, 1999). Efforts were made to improve the activity of immobilized enzymes by mixing alkyl-substituted silanes in specific ratio, and introducing polymers or phyllosilicates into sol-gel matrices (Branyik *et al.,* 2000; Marxer & Schoenfisch, 2005; Li et al., 2006b). An innovative immobilization procedure was developed for intercalation of enzymes into dispersed phyllosilicates which were cross-linked with silicates resulting from the hydrolysis of tetramethyl orthosilicate (TMOS) (Shen & Tu, 1999). Immobilized horseradish peroxidase in the cross-linked phyllosilicate exhibited a similar or higher activity than the free enzyme and similar kinetic properties to the free enzyme and good storage stability (Shen & Tu, 1999). Because of the hydrophilic and hydrophobic sites on the silicates layers, such phyllosilicate sol-gel matrix was a good choice for lipase immobilization (Hsu *et al.*, 2000). The activity of the phyllosilicate sol-gel-immobilized lipase was dependent upon the type of salt and volume ratio of phyllosilicate clay to TMOS used. Increasing the concentration of TMOS caused a decrease in the activity of immobilized enzyme. In contrast,

increased additive salt (*e.g.,* NaF) content caused the activity of the immobilized enzyme to increase (Hsu *et al.,* 2000). The concentration of TMOS and salt affect the network pore size of the gel formed, which is not yet clearly understood (Hsu *et al.,* 2000).

2.2.4 Encapsulation

Encapsulation of enzymes can be achieved by enveloping the biocatalysts within various forms of semipermeable membranes, so that large protein or enzymes cannot pass out of / into the capsule, but small substrates and products can pass freely across the semipermeable membranes (Fig. 2.1) (Bickerstaff, 1997a). Many materials have been used to construct microcapsules, including natural polymers and synthetic polymers. Boadi and Nenfeld (2001) reported that alginate, chitosan, carrageenan and pectin gel matrices were used for encapsulation of tannase for the hydrolysis of tea tannins. Another natural polymer, gum Arabic, was used by Ramakrishnan *et al.,* (2006) as encapsulation material for endoglucanase for potential application in detergent and textile industries. The natural polymers are being favoured, because they are natural, non-toxic and biodegradable, and considered environmentally friendly to the processing materials (Zhu *et al.,* 2005a; Ramakrishnan *et al.,* 2006). The pore sizes of capsules are controlled by the concentration of the polymers. Some of them need added cross-linking reagent (*e.g.,* glutaraldehyde) to improve enzyme retention (Boadi & Neufeld, 2001; Zhu *et al.,* 2005a).

Of the synthetic polymers, the biodegradable types, such as polylactide (PLA), poly-*DL*-lactide-colglycodide (PLGA) (Castellanos *et al.*, 2002) and poly-*DL*-lactide-co-poly-(ethyleneglycol) (PELA) (Li *et al.*, 2000) have been investigated and satisfactory results have been obtained. One of the main problems is the protein unfolding and aggregation caused by encapsulation procedures. Li and co-workers (2000) applied two microencapsulation methods which were based on phase separation and emulsion-evaporation techniques, respectively. The results obtained showed that the solvent extraction / evaporation method based on the formation of double emulsion water-in-oil-in-water ($w_1/o/w_2$) benefited the activity retention compared with the phase separation method based on the formation of water-in-oil-in-oil ($w/o_1/o_2$). However, in the $w_1/o/w_2$ system, the emulsification procedures were highly responsible for the enzyme activity loss (Li *et al.*, 2000). This could be avoided in the solid-in-oil-in-water (s/o/w) technique and the protein stability could be improved (Castellanos *et al.*, 2002). The limitation for this s/o/w method is the low encapsulation efficiencies for protein loading (Castellanos *et al.*, 2002). By optimization of the process parameters and micronization techniques for protein powder particles, the encapsulation efficiencies were improved greatly by Castellanos et al. (2002). Hence, the key points for using synthetic polymers for encapsulation enzyme are minimization of encapsulation induced enzyme structural changes and maximization of encapsulation efficiencies.

The encapsulation of enzymes in microenvironments and especially in liposomes has proven to greatly improve enzyme stabilization against unfolding and denaturation (Colletier *et al.*, 2002; Dufour *et al.*, 1996; Vamvakaki *et al.*, 2005;

Vermette *et al.,* 2004). The encapsulation efficiency of enzymes depends on interaction between the enzyme molecules and the lipid bilayers. High encapsulation efficiency could be achieved by manipulation of liposomal lipid composition, or by increasing the lipid concentration (Colletier *et al.,* 2002).

Silica gels are chemically inert, biocompatible and resistant to microbial attack (Kato *et al.,* 2005). Compared with organic polymers, they show higher mechanical strength. Thus, sol-gel silica glass is also another popular support material for enzyme encapsulation. Moreover, the silica matrix constrains the movement of encapsulated proteins, thus the proteins or enzymes could be protected from aggregation and denaturation under severe conditions (Badjic *et al.,* 2001).

2.2.5 Crosslinking

This type of immobilization is support-free and involves joining the enzymes to each other to form a large, three-dimensional complex structure and can be achieved by chemical or physical methods (Fig. 2.1) (Bickerstaff, 1997a). Chemical methods of crosslinking normally involve covalent bond formation between the enzyme molecules by means of a bi- or multifunctional reagent, such as glutaraldehyde and physical crosslinking involves flocculation (Bickerstaff, 1997a). Flocculating agents such as polyethyleneimine, polystyrene sulfonates, and various phosphates, have been used extensively and are well characterized (Bickerstaff, 1997a). Crosslinking is rarely used as the only means of

immobilization because the absence of mechanical properties and poor stability are severe limitations. Crosslinking is most often used to enhance other methods of immobilization, normally by reducing enzyme leakage in other systems. For example, Boadi & Neufeld (2001) used glutaraldehyde as cross-linking reagent to improve enzyme retention when tannase was encapsulated in alginate, chitosan, carrageenan, or pectin gel matrices. Kilinc *et al.,* (2006), reported that porcine pancreatic lipase (EC 3.1.1.3) was immobilized onto chitin and chitosan by adsorption and subsequent crosslinking with glutaraldehyde to improve the properties of immobilized enzyme (*e.g.,* reusability, storage and operational stability).

2.2.6 Comparisons between different methods

It can be deduced from the information above that each method has its own advantages and drawbacks. Thus, it is hard to make a decision as to which method is good and which is bad. It depends on different applications and different aims, so the same enzyme could be immobilized onto different supports with different methods. For instance, glucose isomerase used in producing high-fructose corn syrup (HFCS) has been immobilized by several methods (Bhosale *et al.,* 1996): adsorption on an anion-exchange resin, entrapment of glucose isomerase involving filaments of cellulose acetate, adsorption of specific SiO_2 particles followed by cross-linking with glutaraldehyde, polythyleneimine-treated alumina with glutaraldehyde cross-linked glucose isomerase. Table 2.3 shows

the advantages and disadvantages of different methods (Martin *et al.,* 1998; Thomas *et al.,* 1993; Tembe *et al.,* 2006).

2.3 Applications of immobilized enzymes

Some recent research and development work led to significant advancement in immobilization techniques applicable to industrial processes (Catana *et al.,* 2005). A few proposals have been selected to discuss in more details as illustrative of what might be done with immobilized enzyme in food industry.

2.3.1 Cheese making

In the commercial production of cheese, the controlled curdling or coagulation of milk is accomplished with proteolytic enzymes called rennets (Emmons *et al.,* 2003; de La Fuente *et al.,* 1998). In order to develop a continuous, automated process for cheese production it would be advantageous to use immobilized rennets (Pessela *et al.,* 2004; Ohmiya *et al.,* 1978). In this process, cooled milk (about 15°C) would pass over the immobilized enzyme column where proteolysis of the milk protein, casein, would occur (Pessela *et al.,* 2004). No coagulation actually occurs until the milk is heated. Heating of the enzyme-treated milk would take place in a separate secondary step to induce curdling (Olson & Korus, 1977). The major difficulties encountered in using immobilized rennet are low activity and poor stability of the immobilized enzymes, release of enzyme during continuous operation, column plugging, and microbial contamination (Olson &

Korus, 1977). Pessela et al. (2004) using covalent immobilization and introducing spacer arms (via sugar chains which was oxidized with periodate previously) between support material and enzyme molecules achieved a proper orientation, and thereby improved the stability and activity of immobilized rennet.

2.3.2 Treatment of lactose in milk

The treatment of milk with lactase to hydrolyse the milk sugar (lactose) to readily adsorbed and/or metabolizable sugars (galactose and glucose) is of fundamental importance to relieve individuals that are lactose intolerant. The simplest and probably the cheapest way is to treat the milk with immobilized lactase packed in a column (Pye, 1974). Sun *et al.,* (1999), reported that lactase was immobilized on cross-linked polyacrylamide gel for hydrolysis of lactose. The immobilized enzyme had a wider operational pH range and better thermal stability compared with the free form.

2.3.3 Milk preservation

Milk is a natural, physiologically produced secretion; it contains many antimicrobial enzymes in quantities high enough to be used for milk preservation (Severin & Wenshui, 2005; Touch *et al.,* 2004). However, the availability of substrates is often low. Thus, the lactoperoxidase (LPS) in raw milk requires an exogenous source of hydrogen peroxide (H_2O_2) to kill bacteria (Law & Goodenough, 1995). In many countries, it is illegal to add H_2O_2 directly. Thus, immobilized β-galactosidase and glucose oxidase are a multiple enzyme system

used in this application to achieve the desired effect. Immobilized β-galactosidase converts lactose (in milk) to glucose, which is the substrate of the immobilized glucose oxidase. Glucose oxidase subsequently converts the glucose to *D*-gluconic acid and hydrogen peroxide (Ukeda *et al.,* 1996). Thus, H_2O_2 is generated by these enzymatic reactions for the lactoperoxidase to have enough substrate. When the mixed immobilized enzymes are packed in a column, the system forms the basis of a continuous-flow, cold sterilization unit (Law & Goodenough, 1995; Fox & McSweeney, 1998).

2.3.4 Protein modification

The enzymatic cleavage of casein yields a number of casein phosphopeptides that may promote Ca uptake by intestinal cells because of no Ca precipitation (Kitts *et al.,* 1992; Kitts & Nakamura, 2006). A new kind of Ca-complexing food ingredient (calcium binding of phosphopeptides) was obtained by hydrolysis of α_s-casein and β-casein with immobilized trypsin (Park *et al.,* 1998; Lorenzen *et al.,* 1994). Peptides were prepared by casein hydrolysis using a fluidized bed bioreactor containing immobilized trypsin (Park *et al.,* 1998). The results showed that phosphopeptide fractions have greater Ca^{2+}-binding ability than do the nonphosphopeptide fractions that were derived from tryptic hydrolysis. Although casein phosphopeptides hold promise for use as a food ingredient with the ability to carry Ca, such as infant formula, sports drinks, or special medical food, further information about tryptic casein phosphopeptides, such as flavor characteristics or interrelations with other components in food systems need to be verified (Park *et al.,* 1998).

Limited proteolysis has been applied to many food proteins as a way of changing functionality (Tardioli *et al.*, 2003). The gelation of β-lactoglobulin treated by limited proteolysis with immobilized trypsin was studied by Chen *et al.* (1994) and Huang *et al.* (1994). Limited proteolysis of β-lactoglobulin with immobilized trypsin produced several fractions of the central β-barrel core domain: ovalbumin (Chen *et al.*, 1993). As a result of this limited proteolysis, transparent thermally induced gels were formed rather than the opaque gels normally obtained with ovalbumin. The result showed how limited proteolysis of β-lactoglobulin affects gelling functionality. Using immobilized trypsin to hydrolyze the β-lactoglobulin, it is convenient to control the extent of the reaction (Chen *et al.*, 1993).

Milk proteins have been enzymatically modified, using neutrase and trypsin immobilized on CM-Sephadex C-50, to the extent of 3, 4 and 5% degrees of hydrolysis (Kumar *et al.*, 2000). The neutrase-treated milk and the trypsin-treated milk were used separately to prepare set yoghurt. The set yoghurt prepared from neutrase-treated milk showed an improvement in body, texture and flavor, a faster rate of acid development and a reduction in setting time compared with the control. However, the set yoghurt prepared from trypsin-treated milk showed either a small or no improvement in textural and sensory properties.

2.3.5 Juice mash treatment

Mash treatments with pectolytic enzymes are now essential in fruit juice industry in order to get higher yields of juice in a shorter processing time, to produce high quality aromatic juices and to reduce the amount of waste pomace (Serrat *et al.*,

2004; Essa & Salama, 2002; Dongowski & Sembries, 2001). With this process protopectin and pectin are degraded into galacturonic acid thereby increasing uronic acid and methanol contents, and the total acidity of the product. Mash liquefaction with immobilized pectinases allows automated continuous processes for fruit juice production (Busto *et al.,* 2005; Vailliant *et al.,* 2000). The pectolytic enzyme preparation was immobilized onto anion-exchange resins (Sarioglu *et al.,* 2001; Demir *et al.,* 2001). Kinetic properties of the free and immobilized pectinases were compared with each other and the temperature effect on the biochemical reaction was investigated in batch system studies. With the immobilization techniques, the thermal stability of the enzyme was found to be increased (Sarioglu *et al.,* 2001; Busto *et al.,* 2005). Optimum working temperature was not affected by immobilization (Busto *et al.,* 2005). Sariogu *et al.* (2001) reported the immobilized pectolytic enzyme was used 5 times in carrot puree medium, and the activity loss was found to be only 6.5%. Activity of the immobilized enzyme was quite stable. Besides good stabilization, there was a 17.7% increase in carrot juice yield caused by the immobilized enzyme preparation. It can be concluded from this observation that the immobilization of commercial pectinase aids carrot mash treatment to obtain high yields of carrot juice.

Vailliant *et al.,* (2000), reported the co-immobilization onto chitin of both pectinlyase and endocellulase enzymes for use in fruit juice liquefaction. Enzymes bound to chitin remain highly active at low pH value (*e.g.,* pH 4.0) and at a low temperature (*e.g.,* 45°C), which represents a significant advantage in the

case of application to acid juices. However, the optimum pH and temperature for the free pectinlyase and endocellulase are pH 5.5 and pH 4.4, 55°C and 45°C, respectively.

2.3.6 Modification of oils and fats

The triglyceride and fatty acid composition determine the commercial value of fats and oils. Thus, several studies have been carried out to improve the fatty acid composition of fats and oils (Gomes *et al.,* 2004; Wyss *et al.,* 2006; Garcia-Alles & Gotor, 1998). Transesterification is one of methods for modification of oils and fats. Lipase-catalyzed transesterification, due to enzyme specificity, provides a precise control over incorporation of desired fatty acid at a specific glycerol position, because operations under mild reaction conditions are preferred over harsh and nonspecific chemical catalysis or random physical blending (Cossignani *et al.,* 2004; Fomuso & Akoh 2001; Garcia-Alles & Gotor, 1998). Several industrial applications of lipase transesterification have been reported, such as production of margarine fat from high-laurate canola (Fomuso & Akoh, 2001), acidolysis of soybean oil triacylglycerols to increase oleic acid content (Cossignani *et al.,* 2004), preparation of non-steroidal anti-inflammatory drug (ketoprofen) by transesterification of glucose with the vinyl ester (Wang *et al.,* 2005), modification of rice bran oil to incorporate capric acid (Jennings & Akoh, 2000).

Immobilized enzymes can make enzymes reusable and stable. A number of useful immobilized lipases have been reported and some of these have been

found to be very useful for application of lipase-catalyzed transesterification in lipid processing and modification (Zhu *et al.,* 2005b; Vidinha *et al.,* 2006; Mustranta *et al.,* 1993). Khare and Nakajima (2000) reported that *Rhizopus japonicus* lipase was immobilized onto a celite support. The immobilized enzyme acquired 6034 units of transesterification activity over the native enzyme, which did not show any significant transesterification activity in *n*-hexane. However, there was no improvement in the thermal stability of celite-immobilized lipase. The preparation is shown to be promising for its end application in producing structured lipid. An incorporation of 25% docosahexaenoic acid (DHA) into soybean oil was observed in *n*-hexane media in 24 h with the immobilized lipase (Khare & Nakajima, 2000).

2.3.7 Beer

The presence of diacetyl is highly undesirable for most beers as it gives to the beer a very unpleasant flavor (odor and taste of butterscotch, buttermilk), when present at concentrations higher than 0.02–0.10 mg/L (Kobayashi *et al.,* 2005). During beer fermentation, yeasts excrete α-acetolactate as an intermediate of leucine and valine biosynthesis, which are essential for yeast growth. The α-acetolactate is then converted into diacetyl by a decarboxylation that is slow. Diacetyl is then transformed into acetoin by an enzymatic reaction (diacetyl reductase). Acetoin has a much higher threshold (30–100 mg/L) (Onnela *et al.,* 1996). The rate-limiting step of fermentation is the conversion of α-acetolactate into diacetyl. Consequently, beer maturation is long. Today, in process control,

the level of diacetyl in beer is used as a tool for supervision, particularly as a signal of the end of warm maturation (Suihko *et al.*, 1990).

The use of the enzyme α-acetolactate decarboxylase has been proposed to overcome the diacetyl formation. This enzyme converts α-acetolactate directly into acetoin without the formation of diacetyl (Onnela *et al.*, 1996). The use of α-acetolactate decarboxylase makes it possible to shorten beer primary fermentation until no more maturation is needed in regard to diacetyl (Suihko *et al.*, 1990).

The use of encapsulated α-acetolactate decarboxylase during primary batch fermentation makes it possible to accelerate the total time of beer production (Dulieu *et al.*, 2000). The encapsulated α-acetolactate decarboxylase allows the acceleration of beer fermentation as efficiently as free α-acetolactate decarboxylase. The advantage of immobilized α-acetolactate decarboxylase versus free enzyme is that it is recoverable and reusable, which means process cost reduction.

2.3.8 Biosensor design

A biosensor is an analytical device in which a biologically derived sensing element is in intimate contact with a physiochemical transducer to give an electrical signal (Mellgren *et al.*, 1996). Biocatalysts for biosensors are enzymes, microbial cells, antibodies, lectins, and plant or animal organelles. The immobilization of these materials onto the surface of a transducer forms the basis

of various biosensors (Fig.2.2). Biosensors are increasingly becoming practical and useful tools in medicine, food quality control, environmental monitoring and research (Rosooly & Rosooly, 1999; Nedelkov *et al.,* 2000; Basu *et al.,* 2005; Yildiz *et al.,* 2005; Yildiz & Toppare, 2005; Tembe *et al.,* 2006; Fuentes *et al.,* 2006). In principle, they can be tailored to match individual analytical demands for almost any target molecule or compound that interacts specifically with a biological system. A biosensor makes use of a biological molecule that is immobilized in proximity to a transducer to detect an analyte, and ultimately transduces the chemical signal produced by the interaction into a measurable response, most often an electronic signal (Marks *et al.,* 2002; Scouten *et al.,* 1995).

The immobilization of biomolecules permits the reuse of costly biological molecules, and allows a significant simplification of the analytical apparatus. Enzymes are by far the most commonly used biological components in biosensors (Murphy, 2006). Furthermore, electrochemical transduction is the most popular signaling method, with amperometry the favored configuration (Eshkenazi *et al.,* 2000; Tang *et al.,* 2005; Tembe *et al.,* 2006; Li *et al.,* 2006a). Oxido-reducatases and amperometric electrodes are the best combination, since an enzymatic reaction with substrates is easily and sensitively measured by electrochemical means (Stoica *et al.,* 2006; Murgida & Hildebrandt, 2005). The instrumentation required for amperometric analyses is inexpensive and simple, and is sensitive over a very wide concentration range. In addition, colored or turbid samples, which would be problematic in spectroscopic analyses, do not

cause interference in electrochemical analyses (Murphy, 2006; Goral *et al.,* 2006; Tizzard *et al.,* 2006).

A critical step in the development of biosensors is effective enzyme immobilization, while maintaining free diffusion of substrates and products into and out of the enzyme layer. The entrapment of the enzyme behind a membrane is often a straightforward process, particularly for the immobilization of living cells or crosslinking enzymes or proteins. Even so, the choice of semipermeable membrane may have a significant effect on the sensitivity and background of the resulting signal (Sahin *et al.,* 2005; Yildiz & Toppare, 2005).

Other biosensors are based on covalently coupling an enzyme to a membrane, which is then attached to the sensing area of an electrode (Ukeda *et al.,* 1996; Basu *et al.,* 2005; Marzouki *et al.,* 2005; Yildiz *et al.,* 2005; Salis *et al.,* 2005). In order to enhance enzyme loading, the enzyme can be crosslinked with a protein such as bovine serum albumin to form a thin layer on the support surface. Numerous bifunctional agents are available for this purpose, including glutaraldehyde, which is the most common crosslinking agent (Draisci *et al.,* 1998).

An example of an electrochemical biosensor is the enzyme diamine oxidase (DAO) immobilized onto an electrode surface for the determination of biogenic amines – putrescine, cadaverine, histamine, tyramine, spermidine, spermine, tryptamine (Draisci *et al.,* 1998).Using glutaraldehyde as a crosslinking agent, DAO was immobilized onto a nylon-net membrane. Variations of the amine

content in anchovies during ripening time were measured both with the biosensor and ion chromatography with integrated pulsed amperometric detection (IC-IPAD). The result exhibited the same trend between the biosensor and the IC-IPAD, and demonstrated that the biosensor is a useful tool to monitor the variation of the total amine content in fish during storage (Draisci *et al.,* 1998).

L-amino acid oxidase (LAAO) has been immobilized onto preactivated nylon membrane as an enzyme sensor, which can detect ammonia (Lee & Hun, 1999). The biosensor was applied for monitoring increases in amino acid levels during yeast autolysis. Determination of *L*-amino acids was complete in 3 min. Moreover, the activity of the enzymic membrane was stable for at least 260 assays and did not noticeably decline for 2 months.

Multienzyme immobilization systems allow for the fabrication of biosensors that have greater versatility. More than one enzymes or proteins are immobilized onto the same support, thus, the substrate is converted to final product in one step (Basu *et al.,* 2005). For determination of *L*-glutamate in both food and clinical samples, *L*-glutamate oxidase (L-GLOD) and *L*-glutamate dehydrogenase (L-GLDH) were co-immobilized to make a monosodium glutamate (MSG) biosensor (Fig. 2.3), which is more compact, sensitive, economic and saves time to use for measuring MSG levels (Basu *et al.,* 2005).

2.3.9 Enzyme immobilization applied in macromolecular or solid substrate

Among the main advantages of immobilized enzymes as compared with the corresponding native soluble ones are: ease of reutilization, enhanced stability, and the possibility of controlling and interrupting the reaction by simply separating them from the reaction mixture (Vidinha *et al.,* 2006; Gomes *et al.,* 2004). On the other hand, immobilized enzymes are diffusion-limited and exhibit very low activity toward high-molecular weight substrates. Thus, there are still major limitations for immobilized enzymes in applications involving macromolecular and insoluble particulate substrates, such as starch, cellulose, RNA, DNA (Bickerstaff, 1997a).

Brena *et al.* (1998) reported a new approach for the control and interruption of enzymatic reactions via selective enzyme immobilization. This method using the enzyme immobilization technique captures the enzyme with free thiols by a solid phase containing thio-reactive structures. Figure 2.4 shows the reactive principle. The enzyme bound to the solid phase can be separated from the reaction mixture by filtration or centrifugation.

2.4 Future development of enzyme immobilization

The extensive development of enzyme immobilization techniques which is now occurring appears to be largely devoted to the design of supports which are relatively cheap, inert, have good mechanical and flow properties for use in continuous systems and are capable of being derivatized extensively (Gomez *et al.,* 2006). An additional feature which is receiving attention is the potential for

chemical modification of the supports surface (Gomez *et al.,* 2006). Many of the more promising newer supports have been designed with these features in mind as well as the more obvious aspects of high loading capacity and high surface area. The biocompatible nanomaterials are one of the examples, which have unique advantages in enzyme immobilization (Long & Keating, 2006; Tang *et al.,* 2005). Li *et al.,* (2006b), reported that nanomaterials could retain the activity of enzymes quite well due to their desirable microenvironment, and they could enhance the direct electron transfer between the enzyme's active site and the biosensor electrode. They used ZnO as semiconductor material, which is suitable for the adsorption of low-isoelectric point proteins due to its high isoelectric point (~9.5). In this study, the nano-ZnO was prepared by mixing ZnO and chitosan and formed ZnO / chitosan matrix. The biosensor made by this kind of material was designed for phenol determination with tyrosinase (pI~4.5). The result showed that the linear range for phenol concentration was from 1.5×10^{-7} mol/L to 6.5×10^{-5} mol/L with a detection limit of 5.0×10^{-8} mol/L obtained at signal/noise ratio of 3.

Another recent advance of considerable consequence to the food industries is the immobilization of enzymes to magnetic powders/beads (Liu *et al.,* 2005). The immobilized enzyme on such magnetic beads can be recovered from reaction mixture and products by means of a magnetic field, even when the substrate or product is insoluable (Bozhinova *et al.,* 2004). Such processes are not readily applicable to column operation because of the problems of low flow rates and column plugging (Gaskin *et al.,* 2001). However, they may be readily operated in

continuous stirred tank reactors using enzymes immobilized onto magnetic supports for continuous or batch recovery and reuse of the enzyme (Liu *et al.*, 2005). The presently available magnetic supports are limited for wide application because of their complex preparation, insufficient enzyme loading capacity and high cost for buying material (Liu *et al.*, 2005).

Novel development in preparing stable immobilized enzymes have received considerable attention because of the demands of industry for more economic, effective and stable immobilized enzyme forms. A new immobilization strategy for enzyme on charged supports via electrostatic interactions, even previous modification of enzyme with charged polymer was proposed by Gomez *et al.*, (2006). Invertase as the model enzyme was previously modified by chitosan and further was immobilized onto a support coated with the anionic polysaccharide pectin (Gomez *et al.*, 2006).

The main challenges in enzyme immobilization include retaining catalytic activity and controlling proper orientation of the immobilized enzyme to ensure the access of the substrate to the active site of the enzymes. The key element for achieving oriented immobilized enzyme is to block the active site of enzyme molecules by a competitive ligand such as inhibitor (Zhen *et al.*, 2004). Wang *et al.*, (2006b), reported an approach using electrostatic orientation of enzymes onto charged surfaces. The enzyme molecules were oriented differently on oppositely charged surfaces, with the majority of active sites facing upward on a positively charged surface and downward on a negatively charged surface. This approach

could control the orientation of enzyme molecules immobilized onto support and improve the efficiency of specific interaction between enzyme and support material.

In my research, covalent binding was chosen as immobilization method, and controlled pore glass (CPG) beads as support material. CPG is an inorganic porous material. It has high mechanical strength, good resistance to solvent or microbial attack, and it is reusable, easy to handle (Clare *et al.,* 2001; Bonneil *et al.,* 2000). The use of CPG as an immobilization system has proven to be effective with a variety of enzymes, *e.g.,* trypsin (Migneault *et al.,* 2004; Sear & Clark, 1993; Janoline & Swaisgood, 1982), urease (Limbut *et al.,* 2004), nitrite reductase (Rosa *et al.,* 2002); β-galactosidase (Petzelbauer *et al.,* 2002). Usually, glutaraldehyde is chosen as a bifunctional cross-linking reagent to pre-modified support material (Yamato *et al.,* 2004), because it is able to react rapidly with primary amines, and thus with enzyme molecules (Migneault *et al.,* 2004), although the reaction mechanism of glutaraldehyde with amines is not fully understood (Walt & Agayn, 1994). The enzyme molecules could be covalently immobilized onto the glutaraldehyde pre-activated CPG. Such method provides stability of immobilized enzyme with high enzyme activity, compared with other methods (Limbut *et al.,* 2004). Migneault and co-workers (2004) reported that the covalently immobilized trypsin was used in tryptic peptide mapping and the obtained covalently immobilized trypsin exhibited lower K_m' (180 μM) and higher specific activity (26 TAME U/mg).

Table 2.1 Fundamental considerations in selecting a support and method of immobilization (Adapted from Bickerstaff, 1997a).

Property	Point for consideration
Physical	Strength, noncompression of particles, available surface area, shape/form (beads/sheets/fibers), degree of porosity, pore volume, permeability, density, space for increased biomass, flow rate and pressure drop.
Chemical	Hydrophilicity (water binding by the support), inertness toward enzyme, available functional groups for modification and regeneration/reuse of support.
Stability	Storage, residual enzyme activity, regeneration of enzyme activity, and mechanical stability of support material disruption by chemicals, pH, temperature, organic solvents and proteases defense mechanisms.
Safety	Biocompatibility (invokes an immune response), toxicity of component reagents, health and safety for process workers and end-product users, specification of immobilized preparation for food, pharmaceutical and medical applications.
Economic	Availability and cost of support, chemicals, special equipment, reagents, technical skill required, environmental impact, industrial-scale chemical preparation, feasibility for scale-up, continuous processing, effective working life, reusable support and CRL[1] or zero contamination (enzyme product).
Reaction	Flow rate, enzyme loading and catalytic productivity, reaction kinetics, side reactions, multiple enzyme system, batch, CSTR[2], PBR[3], FBR[4], ALR[5], and so on; diffusion limitations on mass transfer of cofactor, substrates and products.

[1]CRL: calculated risk level, [2]CSTR: continuous stirred tank reactor, [3]PBR: packed bed reactor, [4]FBR: fluidized bed reactor, [5]ALR: airlift reactor.

Table 2.2 Common supports for covalent binding

Supports	Reference
Natural supports	
Polysaccharide	
Agarose	Kim *et al.*, 2001
Cellulose	Tiller *et al.*, 1999 Turner *et al.*, 2005
Dextran	Giacomini *et al.*, 2001
Chitin and chitosan	Vaillant *et al.*, 2000 Shi *et al.*, 2006 Gomez *et al.*, 2006
Inorganic support	
Porous glass	Clare *et al.*, 2001 Limbut *et al.*, 2004 Kartal & Kilinc, 2006 Janolino & Swaisgood, 1997
Porous silica	Soares, *et al.*, 2001; Sasaki *et al.*, 2001
Synthetic supports	
Acrylamide-based polymers	Bryjak & Kolarz, 1998
Methacrylic acid-based polymers	Guoqiang *et al.*, 1995
Styrene-based polymers	Urek & Pazarlioglu, 2004 Ge & Zhang, 1993 Thomas, *et al.*, 1993

Table 2.3 Advantages and disadvantages of enzyme immobilization methods (Adapted from Scouten *et al.*, 1995)

Method	Advantages	Disadvantages
Covalent Binding	Stable enzyme-support complex, leakage of enzyme is very unlikely, ideal for mass production and commercialization	Complicated and time-consuming; possibility of activity losses due to the reaction involving groups essential for the biological activity.
Adsorption	Simple, mild conditions, less disruptive to enzyme protein	Enzyme linkages are highly dependent on pH, solvent and temperature; insensitive.
Entrapment/ Encapsulation	Universal for any enzyme, mild procedure	Large diffusional barriers, loss of enzyme activity by leakage, possible denaturation of the enzyme molecules as a result of free radicals.
Crosslinking	Simple procedure, strong chemical binding of the biomolecules; widely used in stabilizing physically adsorbed enzymes.	Difficult to control the reaction, requires a large amount enzyme, the enzyme (protein layer has a gelatinous nature lack of rigidity), relatively low enzyme activity.

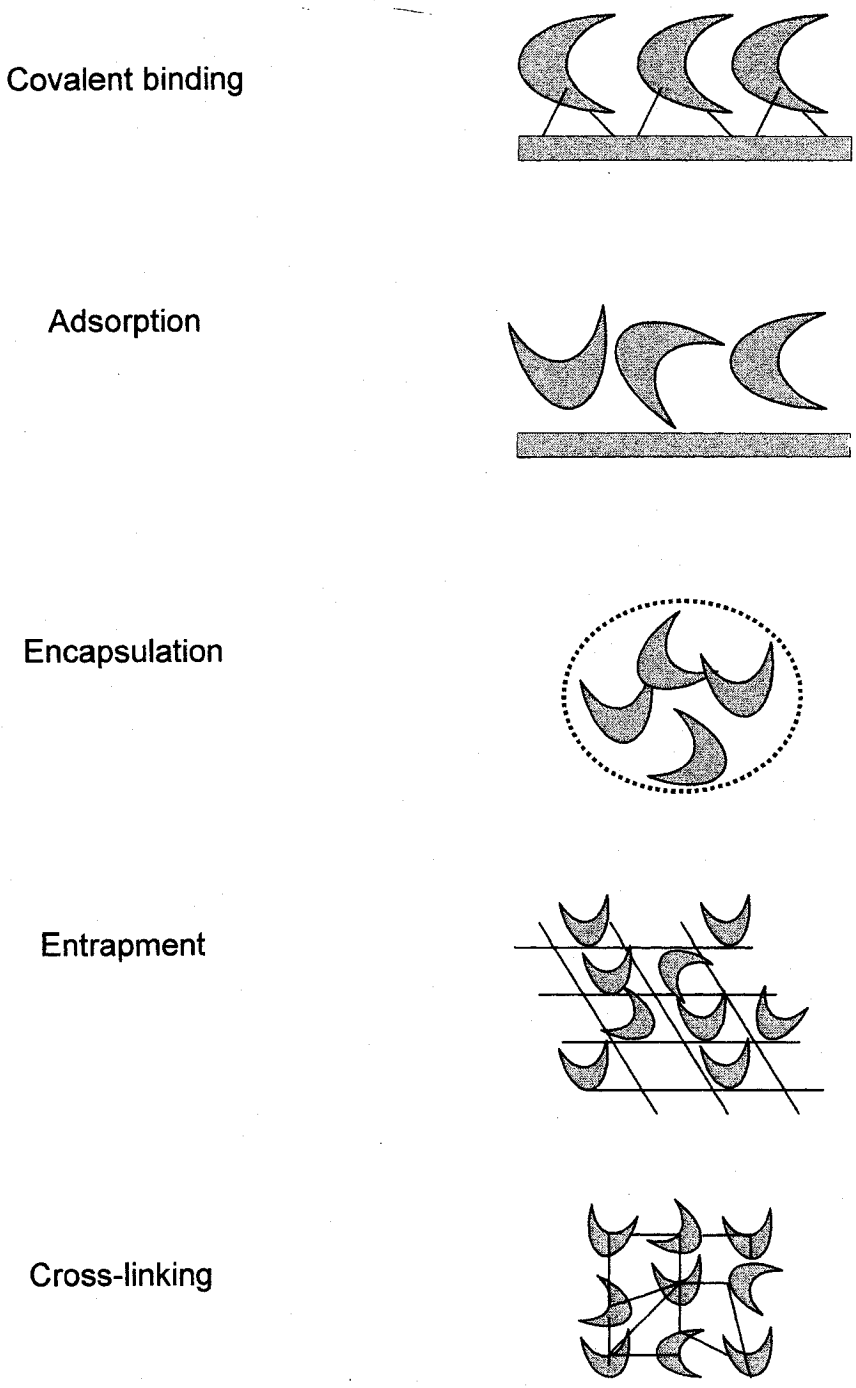


Fig. 2.1: Main methods of immobilization (Adapted from Bickerstaff, 1997a).

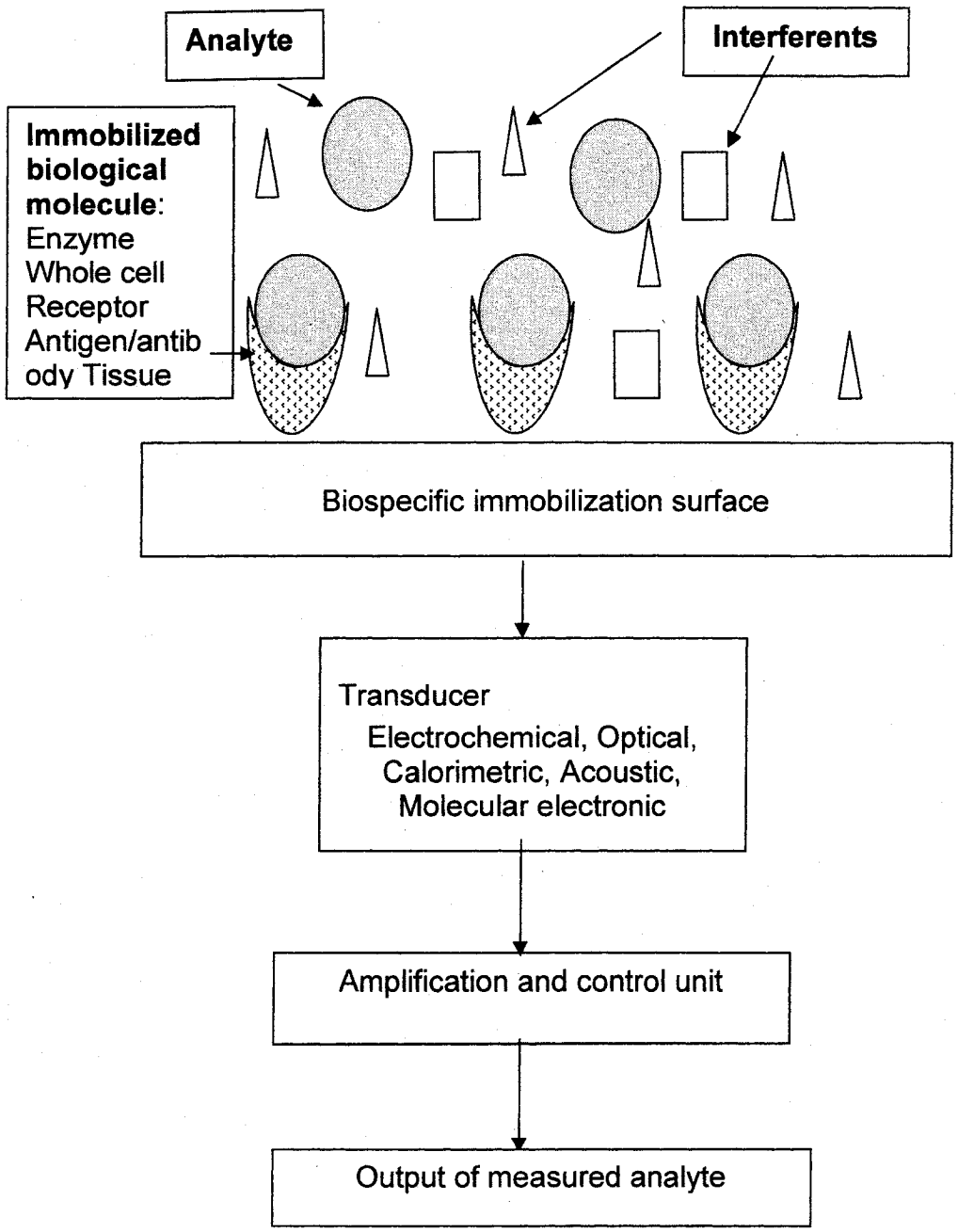


Fig 2.2: Diagram of a biosensor device (Adapted from Scouten *et al.*, 1995)

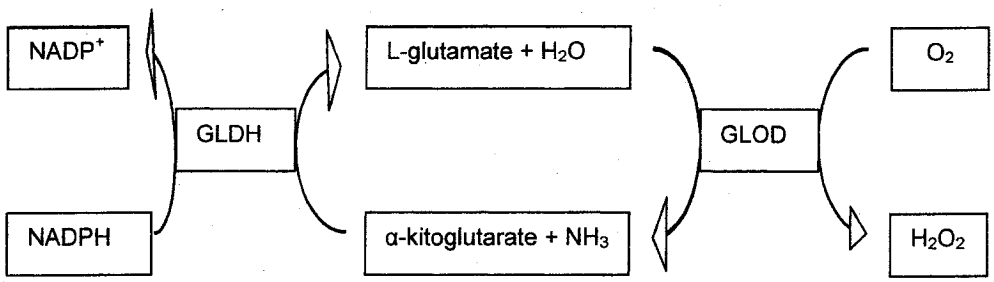


Fig. 2.3: Reaction for determination monosodium glutamate (Adapted from Basu *et al.*, 2005).

$$A: \quad Enz + SPDP \longrightarrow Pyridyl - dithio - Enzyme \xrightarrow{DTT} Thiol - Enzyme$$

$$B: \quad Substrate + Enz - SH \longrightarrow \mathrm{Pr}oduct + Enz - SH$$

$$C: \quad \mathrm{Pr}oduct + Enz - SH \xrightarrow{TSI-Agarose} \mathrm{Pr}oduct + TSI - Agarose - Enz$$

Fig 2.4: Enzyme thiolation and subsequent adsorption as a tool for reaction control (Adapted from Brena *et al.*, 1998).

(A) Introduction of 2-pyridyldithio-propionamide moieties in the protein by reaction with SPDP and reaction of *de novo* introduced 2-pyridyldithio-groups to thiols with DTT; (B) enzymatic reaction catalyzed by the thiolated enzyme; (C) selective immobilization of thiolated enzyme to TSI-agarose. (SPDP: *N*-succinimidyl-3-(2-pyridyldithio) propionate; DTT: 1,4-dithio-*DL*-threitol)

CHAPTER III

IMMOBILIZATION OF BOVINE TRYPSIN ONTO CONTROLLED PORE GLASS (CPG)

Connecting Statement

The utilization of enzyme immobilization technology is a possible means for reusing enzymes and reducing food processing cost for the food industry. Trypsins and other proteases have received more attention by the food industry because they are used extensively in food processing. For example, approximately 50% of the enzymes used as processing aids in food industry are protein hydrolases. The study reported here is on bovine trypsin immobilization by covalent binding method to serve as a model, and also provide some background information for use in the study of fish trypsin immobilization in subsequent studies.

Note: This chapter constitutes the text of a paper to be submitted for publication as follows:

Li, D. and Simpson, B.K. Trypsin Immobilization onto Controlled Pore Glass (CPG)

Contribution of co-author(s): Simpson, B.K. (research / thesis supervisor) gave instructions to the candidate during research, provided financial support for the experiment, and edited the manuscript of this chapter.

Role of candidate: Candidate designed, conducted, analyzed and reported all the experiments presented.

ABSTRACT

Bovine trypsin was immobilized onto controlled pore glass (CPG) beads by covalent binding using glutaraldehyde as a cross-linking reagent. Increasing the concentration of the enzyme solution resulted in an increase in the amount of enzyme protein bound to the support material. The concentration of trypsin used ranged from 1 mg/ml to 6 mg/ml, and the final amount of protein bound to the support materials increased from 6.22 mg / g CPG beads to 25.78 mg / g CPG beads. The effect of pH on immobilization was studied as well. At pH 9, around 60% enzyme protein incubated with the CPG was immobilized onto the CPG and the immobilized trypsin activity was determined as 0.265 BAPNA U / g CPG beads. For the pH range investigated (pH 3.0 to pH 11.0), the pH stability of the immobilized enzyme was shifted to a relatively higher pH value (pH 9) compared to that of the free soluble form of the enzyme (pH 3 & 5). Optimum conditions for trypsin immobilization were established as: trypsin solution concentration of 4 mg/ml, incubation time for 21 h, and reaction pH at pH 9. The immobilized enzyme was evaluated for its capacity to extract carotenoproteins from shrimp shell. After 11 re-uses, the immobilized enzyme retained about 77% of its initial activity, and the total yield of the product from the same immobilized trypsin of 11 re-uses was 4.3 times higher than a single use of the same amount of the free enzyme.

Key words: trypsin, enzyme immobilization, controlled pore glass (CPG)

INTRODUCTION

Enzyme immobilization is a technique used to improve the stability of enzymes, facilitate recovery and reuse of enzymes, as well as reduce the cost of enzyme applications (Saroglu *et al.*, 2001; Demir *et al.,* 2001). In general, immobilization can be achieved by physical methods or chemical methods. Because of the leakage and instability of the physical method, researchers have focused more attention on the chemical methods (Bickerstaff, 1997a). Although, chemical methods are complicated and time-consuming, the enzyme molecules could be bound to the support material more firmly.

Controlled pore glass (CPG) is an inorganic porous material. The main compound of CPG is SiO_2. It has high mechanical strength, good resistance to solvent or microbial attack, and it is reusable, and easy to handle (Clare *et al.,* 2001). Binding of enzymes to CPG could be achieved by various reagents. Usually, glutaraldehyde-CPG and succinylated-CPG were prepared (Janolino & Swaisgood, 1982; Sears & Clark, 1993; Migneault *et al.,* 2004). Both of them showed that enzymes could be bound to the support effectively and showed more stable properties compared with the free form.

Glutaraldehyde is a widely used bifunctional crossing linking reagent, which reacts with lysine residues on the exteriors of the protein (Draisci *et al.,* 1998; Limbut *et al.,* 2004). It has two potential aldehyde groups that can react, and it readily polymerises to form a backbone with a number of pendant aldehyde

groups (Fig 3.1) (Walt & Agayn, 1994). Petzelbauer *et al.,* (2002), reported that β-galactosidase immobilized onto CPG with glutaraldehyde exhibited as high as 80% of the activity of the native enzymes. Acid phosphatase immobilized onto the cross-linked chitosan beads with glutaraldehyde retained about 80% of the original activity if the concentration of the cross-linking agent was high enough (greater than 5000 mg/l, Juang *et al.*, 2001).

Bovine trypsin was chosen as a model enzyme, because it is a well characterized enzyme and is also widely used in the food industry. Immobilization of bovine trypsin has also been studied to some extent (Migneault *et al.,* 2004; Ge & Zhang, 1993; Suma *et al.*, 1995). For example, immobilized bovine trypsin was used to hydrolyze casein in order to improve the desirable functional properties of this food protein (Pedroche *et al.,* 2004; Haque & Mozaffar, 1992; Mozaffar & Haque, 1992). Because of the relative ease of separation of the immobilized enzyme from a reaction mixture, it is easier to control the extent of hydrolysis, to avoid excessive proteolysis that results in bitter peptides and loss of desirable texture in cheese (Haque & Mozaffar, 1992).

The study on bovine trypsin immobilization could promote the progress on the application of enzyme immobilization in the industry, and also provide some background information for use in subsequent studies on fish trypsin immobilization.

MATERIALS AND METHODS

Materials

Controlled pore glass beads (CPG2000A, surface area 11 m^2/g bead, mesh range 80/120) were purchased from CPG Inc, Lincoln Park, NJ. *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA), dimethyl sulfoxide (DMSO), 3-aminopropyltriethoxysilane, glutaraldehyde, bovine pancreas trypsin (type III), ethylenediaminetetraacetic acid (EDTA), glycine, and nitric acid were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Sodium borate, sodium acetate, sodium chloride, hydrochloric acid, calcium chloride, sodium hydroxide, monobasic / dibasic potassium phosphate, monobasic / dibasic sodium phosphate were purchased from Fisher Chemicals (Nepean, Ontario, Canada). Shrimp shells were obtained from Les Fruits de Mer (Matane, Quebec, Canada)

Preparation of controlled pore glass (CPG)

In preparation for derivatization, it is very important to clean CPG beads to generate the maximum number of silanol groups. The procedure of Janolino & Swaisgood (1997) was used to clean the CPG beads. This was done by heating CPG beads in boiling water bath with concentrated nitric acid (1:2 w/v) for 60 min. For glass beads that had been used previously, the beads were heated in a furnace at 600°C for 24 h before incubation with nitric acid. The beads were subsequently washed extensively in a large, coarse fritted-glass under suction

with distilled water (approximately 1:20 w/v CPG to water), until the pH of the washings was neutral.

Synthesis of aminopropyl CPG

The method of Janolino & Swaisgood (1997) was used for the synthesis of aminopropyl CPG. A 10% (v/v) aqueous solution of 3-aminopropyltriethoxysilane was prepared and the pH was adjusted to 4.0 with 6 M HCl. Cleaned CPG was added to 3 vol of the 3-aminopropyltriethoxysilane solution. The mixture was degassed under vacuum to ensure that all of the pore volume is filled with the reagent and then incubated at 70°C for 3 h with occasional mixing. The excess reagent was decanted, and the wet beads were placed in an oven at 100°C for 12 h for direct polymerization. The fine generated by mixing the beads was removed with a large volume of distilled water. The beads were washed with distilled water (about 10:1 v/w water to CPG) on a fritted-glass filter under suction, and dried for 12 h in an oven at 80°C.

Synthesis of glutaraldehyde-CPG

The synthesis of glutaraldehyde-CPG was based on the method of Sear and Clark (1993). Aminopropyl glass beads were washed with distilled water (about 1:100 w/v CPG to water) and suspended in 10 mM sodium phosphate buffer, pH 7.3, containing 10% glutaraldehyde (1:10 w/v CPG to buffer). The slurry was rotated end over end at room temperature (25°C) for 20 min, and then washed

over a Millipore filter (0.45 μm pore size) with distilled water (around 1:500 w/v CPG to water).

Immobilization of enzyme

The procedure of enzyme immobilization was based on the method of Sears and Clark (1993). The activated glass beads were added to 10 mM sodium phosphate buffer, pH 7.3, (1:10 w/v CPG to buffer) containing 4 mg enzyme / ml buffer. The enzyme solution was recycled by pump (Micro tube pump MP-3, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and incubated with the glutaraldehyde-CPG beads for 21 h at 4°C. The CPG beads were subsequently washed with 100 mM sodium borate / 1 M sodium chloride, pH 8.5, followed 100 mM sodium acetate / 1 M sodium chloride, pH 4.75 (1:250 w/v CPG to buffer). The washings were repeated for 4 times as above and finally with 10 mM sodium phosphate buffer, pH 7.3 (1:1000 w/v CPG to buffer). The damp beads were collected and lyophilized to dryness (LYPH-LOCK 12 freeze dry / shell freeze system, Labconco, Kansas City, Missouri).

Activity determination of the immobilized trypsin

The activities of the free trypsins were determined by the method of Erlanger *et al.*, (1961) in terms of BAPNA units. For the immobilized trypsin, the method was modified as follows. 0.5 g of dry immobilized trypsin in a test tube was heated in a water bath at 25°C for at least 15 min. Five mililiters of 1 mM BAPNA solution in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02 M $CaCl_2$) were then added to the

immobilized trypsin. The test tube was shaken in a water-bath shaker (130 rpm, Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min at 25°C. The suspension was centrifuged at 12,000 g for 1 min at 25°C (Biofuge 13, Heraeus Instruments, Baxter Canlab, Mon-Royal, QC), and the absorbance of the supernatant was measured with an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at 410 nm. One BAPNA unit of activity was defined as $\Delta A_{410nm/min}$ x 5 x 1000 / 8800, where 8800 is the extinction coefficient of *p*-nitroaniline and 5 is the total volume of reaction mixture.

Determination of protein binding by CPG

The protein concentrations of the enzyme solutions were determined by measuring their absorbances in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at both 260 nm and 280 nm using the formula below (Dunn, 1989):

$$protein\,(mg/ml) = 1.55\,A_{280} - 0.76\,A_{260} \qquad (3\text{-}1)$$

The amount of bound protein was calculated as the difference between the concentration of protein in solution before and after immobilization.

Effect of pH on trypsin immobilization

The effect of pH in the range of 3 – 11 on the extent of trypsin immobilization and its storage stability was studied based on the methods of Vaillant *et al.*, (2000).

The buffers used were 10 mM citric acid – NaOH buffer for pH 3 and pH 5; 10 mM potassium phosphate buffer for pH 7; 10 mM glycine – NaOH buffer for pH 9; and 10 mM disodium hydrogen phosphate – NaOH buffer for pH 11. Immobilized trypsins were prepared under different pH conditions (pH 3 – 11). 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate to evaluate trypsin activity. The immobilized trypsins were assessed for the amount of bound protein and the activity of the immobilized trypsin. The storage stability of the immobilized trypsin under different pH was studied by keeping the immobilized enzymes at 4° C in the buffers with different pHs and their activities were measured each two weeks.

Determination of optimum conditions for trypsin immobilization

To determine the optimum conditions for trypsin immobilization, enzyme solution concentration and immobilization period were chosen as the factors. The trypsin solution concentrations used were 2 mg/ml, 4 mg/ml and 6 mg/ml, and the immobilization times used were 14 h, 21 h and 28 h (Table 3.1). The reaction pH and temperature were pH 9 and 4° C, respectively. The assays were operated according to the experimental plan shown in Table 3.2. The extent of immobilization was evaluated by amount of bound protein and relative activity of the immobilized enzyme. The detail results were shown in Fig. 3.9 and Fig. 3.10.

Operational stability (Recovery of carotenoproteins with immobilized enzyme)

To do this, the immobilized enzyme was used to aid the extraction of carotenoprotein from shrimp shell, based on the procedure by Ya *et al.*, (1991), and the yields of extracted pigment protein complexes were measured. The crushed shrimp shells were incubated with immobilized trypsin in 0.1 M EDTA buffer, pH 7.7 at room temperature (25°C) for 6 h with continuous stirring. The mixture was filtered with cheese cloth, and the filtrate was collected and adjusted to pH 4.5 with 6 M HCl, and the filtrate was left at room temperature (25°C) for 5 h to sediment. The mixture was centrifuged at 6,000g in a J2-21 centrifuge (Beckman Coultier Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C. The precipitate was collected and freeze-dried (LYPH-LOCK 12 freeze dry / shell freeze system, Labconco, Kansas City, Missouri). The operational stability was indicated by the yield of extracted pigment from shrimp shell by using the same immobilized trypsin for several times.

RESULTS AND DISCUSSION

Binding of bovine trypsin onto CPG

Table 3.3 shows the relationship between trypsin concentration and the amount of protein bound to the support. The data indicate that increasing the concentration of the trypsin resulted in an increase in the amount of protein bound to the support material. The concentration of the trypsin used ranged from

1 mg/ml to 6 mg/ml, and the final amount of protein bound to the support increased from 6.21 mg/g CPG beads to 25.78 mg/g CPG beads.

A model for the chemical binding process was proposed by Langmuir in 1916 (Mead, 1981). According to the Langmuir theory, the relationship between the trypsin concentration (C) and the amount of protein bound to the support (y) is established as:

$$\frac{C}{y} = \frac{C}{y_m} + \frac{a}{y_m} \tag{3-2}$$

Where, a is binding / unbinding equilibrium constant, which is related to bonding energy; y_m is the maximum monolayer binding capacity (Mead, 1981).

If the experimental data are in accord with the Langmuir theory, a plot of C/y versus C should yield a straight line. As shown in Fig. 3.2, the intercept on y axis is identified by a/y_m and the slope as $1/y_m$.

$$Slope = \frac{1}{y_m} = 0.0158\ g/mg \tag{3-3}$$

$$Intercept = \frac{a}{y_m} = 0.1232\ g\ beads/ml \tag{3-4}$$

So: y_m = 63.29 mg/g bead, a = 7.80 mg/ml. It means 63.29 mg protein could be bound to one gram of bead.

The maximum amount (63.29 mg/g bead) is much more than the actual amount (25.78 mg/g bead) bound to the support in this study. A possible explanation for the reduced amount of binding is that the bound protein could block the pore openings, so that other proteins were unable to get into the pores (Hudson *et al.*, 2005). On the other hand, the tortuosity (τ) affects the accessibility of the pore to the enzyme molecules (Fig.3.3). The higher tortuosity of the support possesses, the harder the accessibility for the enzyme molecules (Backer & Baron, 1993; Scharer *et al.*, 1992).

From the maximum amount of protein bound to support, the surface area for the support could be estimated. One gram of bead could be covered by 63.29 mg of trypsin molecules. The molecular weight for trypsin is ~ 23,800 Da, so the number of molecules that could be arranged onto the support for monolayer formation could be calculated by:

$$Number\ of\ molecules\ for\ monolayer = \frac{63.29\times10^{-3}}{23{,}800} \times 6.02\times10^{23}$$

$$= 1.60\times10^{18}\ molecules/g\ CPG\ beads \qquad (3\text{-}5)$$

where 6.02×10^{23} is the Avogadro's number, which means the number of molecules in one mole.

Trypsin is a globular molecular with diameter 38 Å (Goradia *et al.*, 2005). So the area covered by one molecule is

$$\pi \times (\frac{3.8\times10^{-9}}{2})^2 = 1.14\times10^{-17}\ m^2 \quad (3\text{-}6)$$

Therefore, the total surface area for one gram of support is

$$1.60\times10^{18} \times 1.14\times10^{-17} = 18.24\ m^2 \quad (3\text{-}7)$$

which is higher than the data (11 m^2/g bead) from the product catalog book.

The Langmuir theory is based on two assumptions as follows: (i) the molecules ultimately form a monolayer over the surface of the support material; and (ii) the surface of the support is even, with no interactions between the protein molecules (Barrow, 1979). This kind of binding is considered as an ideal binding, but the actual situation with real molecules may be more complex than the ideal conditions, as exemplified by the difference between the calculated surface area and actual surface area.

If the binding obeys the monolayer theory, the calculated surface area should be similar with the actual surface area. However, because of the existence of the multilayer binding of protein molecules, the calculated surface area is higher than the actual surface area. It can be explained by Fig.3.4. If the protein molecules occupy the surface of the support material with monolayer binding and the surface of the support material is saturated with molecules, the calculated surface area of the support material is the sum of areas of all the protein molecules occupied. In the actual binding, however, because of the multilayer binding, some

protein molecules do not attach to the support material directly (attach with other protein molecules), so do not occupy any surface of the support material. If the surface area of support material is calculated according to the number of bound protein molecules, the calculated surface area of the support material must be greater than the actual surface area.

The R^2 value (0.729) derived from the Langmuir model (Fig. 3.2) suggests that the actual binding could be different from the theoretical or expected binding. Perhaps, the difference is due to the interactions between the bound enzyme molecules, thus, resulting in the formation of multilayer of protein molecules, which is against one of assumptions of the Langmuir theory (Li *et al.,* 2004).

A fairly satisfactory empirical isotherm, which can be applied to bindings of gases with considerable success but has been used principally for binding from solution, is the Freundlich model (Barrow, 1979). According to this model, if y is the weight of solute bound per gram of the binding agent and C is the concentration of the solute in the solution, then the empirical relation is:

$$y = kC^{\frac{1}{n}} \qquad (3\text{-}8)$$

where k and n are empirical constants, which are related to binding capacity and intensity, respectively (Van Bladel & Moreale, 1977). k shows the amount of solute bound to one gram of the binding agent, when the solute concentration is 1 mg/ml. $1/n$ shows how fast the amount of solute bound to one gram of the

binding agent increases with increasing solute concentration. The equation is conveniently used in the logarithmic form as:

$$\ln y = \ln k + \frac{1}{n}\ln C \qquad (3\text{-}9)$$

The above is a straight line equation, and as shown in Fig. 3.5, a considerably good correlation is obtained (R^2 = 0.9412) from a plot of ln y versus ln C. Thus, the Freundlich empirical model fitted the practical conditions better than the Langmuir theoretical model.

Based on the above, the possibility of multilayer binding must be considered for the binding of proteins to support materials in practice, because of uneven conditions of the support material and potential interactions between protein molecules (Li *et al.,* 2004).

A third binding model, the Temkin model, also describes the relationship between the protein solution concentration (C) and the amount of protein (y) bound to the support material. The Temkin model, however, is only suitable for chemical binding of protein molecules and support materials (Li *et al.,* 2004):

$$y = \alpha + \beta \ln C \qquad (3\text{-}10)$$

where α and β are constants (Mead, 1981). The Temkin model as shown in Eq. (3 – 10) is a straight line equation and a plot of y against ln C is linear (Fig. 3.6). Similar to Freundlish model, α shows the amount of solute bound to one gram of

the binding agent, when the solute concentration is 1 mg/ml. β shows how fast the amount of solute bound to one gram of the binding agent increases with increasing solute concentration.

The data presented in Fig. 3.6 is based on the Temkin model, and the high R^2 value (R^2 = 0.9842) indicates that there was chemical binding between the enzyme molecules and the support material (Li *et al.*, 2004), where the most plausible reactants involved in the binding are the aldehyde groups on the surface of the support and primary amino groups on the surface of the enzyme molecules (Walt & Agayn, 1994, Fig. 3.7).

The effect of pH on trypsin immobilization

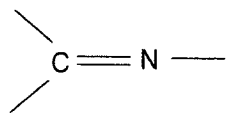
At different pH conditions, various amounts of proteins were bound to the support materials and the immobilized enzymes showed different activities (Table 3.4). The storage stability also varied with different pHs (Fig. 3.8). The data presented in the figure show that the immobilized trypsin exhibited higher stability compared with the free forms.

The above results indicated that more immobilization and the most activity occurred at pH 9, even though it had relatively low specific activity at pH 9 (Table 3.4). Also, the immobilized enzyme at pH 9 retained a higher activity for a longer time than the others. These observations suggest that the enzyme is relatively more stable at pH 9, compared to the other pH values investigated. In contrast, the free enzyme was unstable at higher pH values (*e.g.*, pH 9 and pH 11, Fig.

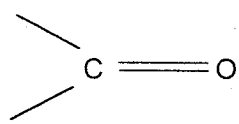
3.8), but most stable at lower pHs (*e.g.*, pH 3 and pH 5, Fig. 3.8). For example, the free trypsin lost almost all its activity within 1 month of storage at pH 9 and 11, unlike the immobilized enzyme which maintained relatively high activity (95% of initial activity) up to 10 weeks. The storage stability is a very important parameter for immobilized enzyme applications, because it determines the reusability of the immobilized enzyme.

The pH optimum and the pH stability of the immobilized enzyme were shifted to higher pH values compared with the unbound enzyme. It could be explained by the reaction pathway between aldehyde groups from the modified support and the amino groups on the surface of enzyme molecules (Walt & Agayn, 1994). One reaction that is possible is the simple formation of a Schiff base (Walt & Agayn, 1994, Fig.3.7).

Aldehyde groups react with primary amine to give an imine



which is called necleophilic addition reaction (Migneault *et al.*, 2004). Reaction rate depends on the concentration of



and the RNH_2 in solution. Accordingly, the solution should be basic or neutral to prevent formation of

$$RNH_3^+$$

However, the solution should be acidic enough to cause the proton transfers, which are accelerated by the presence of an acid (Brown, 1975). Therefore, at a high pH, the reaction is slow because the proton transfers are slow, and at a low pH, the reaction is slow because the concentration of RNH_2 is small. There is an intermediate pH at which the rate is a maximum (Brown, 1975). The stability of the Schiff bases decreases with decreasing pH as previously observed (Zabinski & Toney, 2001). At more alkaline pH (*i.e.,* 9 - 10), Schiff base formation occurs rapidly and with high efficiency, resulting in greater incorporation of enzyme. Thus, in this study, at pH 9, more protein bound to the modified support, and the immobilized enzyme displayed more activity.

From Fig. 3.8, it could be noticed that the immobilized enzyme at pH 3, 5 and 7 showed relatively high specific activities, even though they did not have too much bound protein. However, with increasing storage period, the immobilized enzyme at those pHs could not keep such high activities. It indicated that the immobilized enzymes were not stable at those pHs. On the other hand, at pH 9, the immobilized enzyme bound much more protein than the immobilized enzyme at other pHs, but it showed lower specific activity than the other immobilized enzyme at lower pHs. Perhaps, the free bovine trypsin does not exhibit high

activity at high pH, *e.g.*, pH 9, because it has optimum pH at pH 8 (Anonymous, 1972). However, the enzyme-support complex is stable at such pH. Thus, the immobilized enzyme at pH 9 showed lower specific activity with more bound protein, and the immobilized enzyme could keep its activity even a long storage period. On the other hand, more protein bound to the support also is good for the operational stability, because it could prevent the immobilized enzyme from activity loss caused by the leakage of enzyme molecules from support materials.

The optimum pH for immobilization and storage is 9, as well as it is close to the optimum pH of the immobilized enzyme, such condition can make the enzymatic reaction stable and effective, thus, enhance the operational feasibility of the immobilized enzyme. Therefore, pH 9 was chosen as the optimum immobilization condition for further study.

Optimum conditions for trypsin immobilization

The results obtained using different incubation times and different trypsin concentrations to determine the optimum conditions for trypsin immobilization at pH 9.0 are presented in Figs. 3.9 and 3.10. Figure 3.9 shows that the initial trypsin concentrations and the incubation times affect the amount of bound protein. However, at much longer incubation time (up to 21 h) and higher trypsin concentration (up to 4 mg/ml), the increase of protein binding, from 16.96 mg/g beads at trypsin concentration 2 mg/ml, incubation time 14 h to 23.87 mg/g bead at trypsin concentration 4 mg/ml, incubation time 21 h, was more pronounced.

Figure 3.10 indicates that there is an optimum level of activity for trypsin immobilization. Higher or lower trypsin concentrations and/or incubation times resulted in relatively lower activities for the immobilized enzymes. Presumably, the higher trypsin concentrations and longer incubation times caused the enzyme proteins to denature. It is possible that such denatured protein molecules were simply absorbed onto the CPG and did not express any activity. Also, the higher trypsin concentrations and longer incubation times are not economical for industrial application. An incubation time of 21 h and trypsin concentration of 4 mg/ml appeared to be optimal for the immobilized enzyme.

From the above data, the optimum immobilization conditions were selected as 21 h (incubation time), 4 mg/ml (trypsin concentration) and pH 9.0.

Operational stability (Recovery of carotenoproteins with immobilized enzyme)

The operational stability of the enzyme was investigated using an established method developed in our laboratory for the extraction of carotenoprotein from shrimp shell. This method aimed at reducing processing cost while maintaining higher yields was carried out at room temperature (25°C). The variations in the yields may be due to loss of activity (from the immobilized enzyme) and / or fluctuations in ambient temperature. Nevertheless, the relative activity of immobilized enzyme remained quite high (77% of the original activity) even after 11 re-uses (Fig. 3.11). The yield of carotenoprotein for immobilized trypsin was from 4.53% to 3.47% for the first time use till the 11 re-uses and the yield of

carotenoprotein for free trypsin was 9.75% for one time use. Compared with the free trypsin, the overall yield of the product from the same immobilized trypsin for 11 re-uses was several times (*e.g.*, 4.3 folds) higher than the single use of the free form. The cost of enzyme itself is a big part of the production investment, so it is very important to be able to reduce this cost. The reusability of the immobilized enzyme is one of the most important advantages of enzyme immobilization. During the operation, it was observed that the immobilized enzyme could be removed easily from the mixture upon completion of the hydrolysis reaction, and this immobilized form could then be used for the next batch of reactions

CONCLUSIONS

Trypsin was immobilized onto CPG efficiently, about 60% enzyme protein incubated with the CPG was immobilized onto the CPG and immobilized trypsin activity was determined as 0.265 BAPNA U/g CPG beads. Immobilization also altered the characteristics of the free enzyme. For the investigated pH range (pH 3.0 to pH 11.0), the optimum pH stability of the immobilized enzyme shifted to a relatively higher pH value (pH 9) compared to that of the free form of the enzyme (pH 3 & 5). The optimum immobilization conditions for bovine trypsin were obtained with an initial trypsin concentration of 4 mg/ml, incubation time of 21 h, and a pH of 9.0 at reaction temperature 4°C. Immobilized trypsin may be used to facilitate the recovery of carotenoproteins, and the immobilized form of the enzyme could be re-used several times with high activity.

Table 3. 1: Factors and levels of immobilization conditions

Levels	Factors	
	Immobilization period (h)	Enzyme solution[1] concentration (mg/ml)
1	14	2
2	21	4
3	28	6

Note: Reactions were carried out at pH 9 with 10 mM glycine-NaOH buffer, and temperature at 4°C.

[1] Bovine tryspin

Table 3.2: Experimental plan for optimum conditions of immobilization

Treatment number	Immobilization period (h)	Enzyme solution[1] concentration (mg/ml)
1	14	2
2	14	4
3	14	6
4	21	2
5	21	4
6	21	6
7	28	2
8	28	4
9	28	6

Note: Reactions were carried out at pH 9 with 10 mM glycine-NaOH buffer, and temperature at 4°C.

[1] Bovine tryspin

Table 3.3: Effect of enzyme concentration on the amount of protein bound to support

Relation between bovine trypsin concentration and amount of bound protein

Bovine trypsin concentration (mg/ml)	1	2	4	6
Amount of protein bound to support (mg/g bead)	6.21	14.63	23.87	25.78
Standard deviation of amount of protein bound to support	1.40	1.25	1.06	1.07

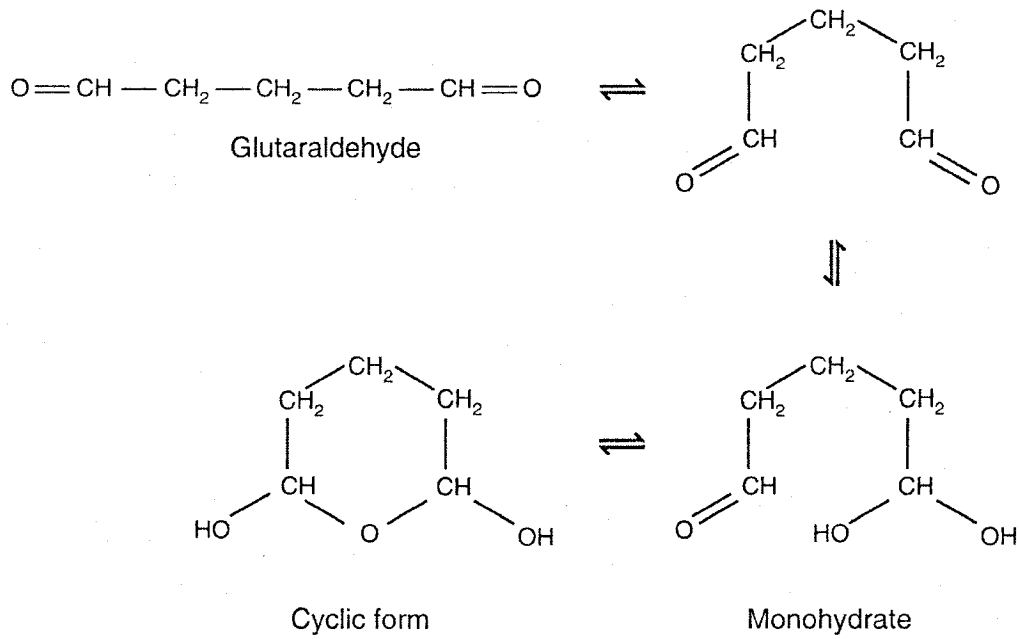
Note: 1. Reactions were carried out at pH 9 with 10 mM glycine-NaOH buffer, and temperature at 4°C.

2. Each value of the amount of protein bound to support is an average of three replicates.

Table 3.4: Amount of bound protein versus immobilized bovine trypsin activity at different pHs

pH	Protein added (mg/g CPG bead) A	Protein bound (mg/g CPG bead) B	Percentage of protein bound (B/A × 100 (%))	Activity of immobilized enzyme (BAPNA U/g CPG bead)	Specific activity (X 10^{-3} BAPNA U/mg bound protein)
3	40	5.76	14.4	0.151	26.24
5	40	5.40	13.5	0.192	35.56
7	40	10.32	25.8	0.198	19.16
9	40	23.87	59.68	0.265	11.10
11	40	13.20	33.0	0.153	11.62

Note: Reaction temperature was at 4°C.



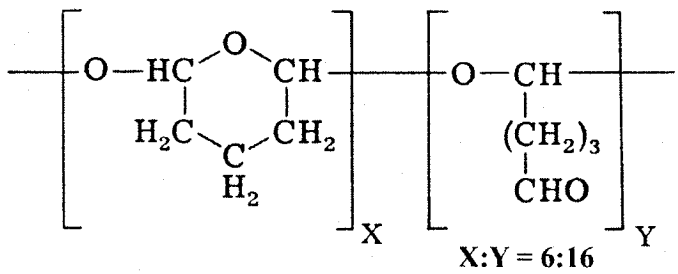


Fig.3.1: The polymerization and formation of glutaraldehyde backbone

(http://www.food.rdg.ac.uk/online/fs916/lect12/lect12.htm, 2005-6-24)

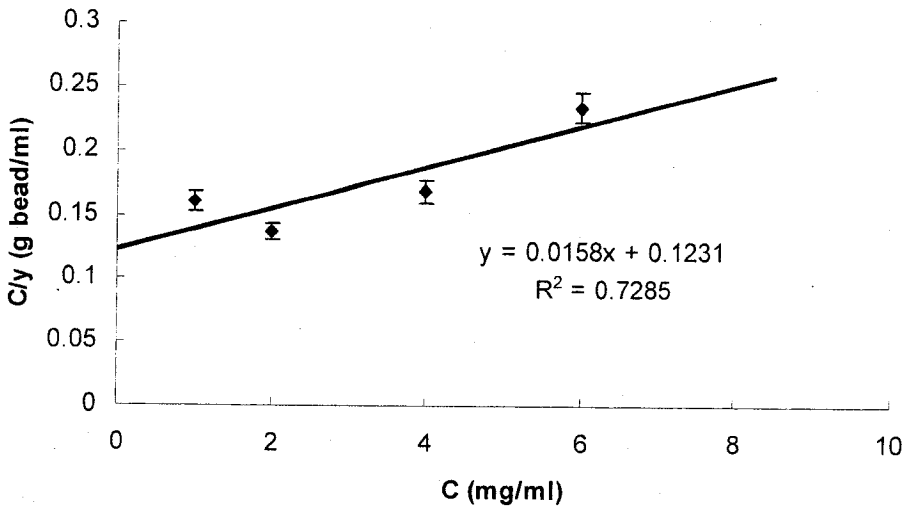


Fig.3.2: Langmuir fitting curve of enzyme binding

Note: 1. C = trypsin solution concentration (mg/ml); y = amount of protein bound to the support material (mg/g bead); R^2 = measure of the fit of the regression line.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

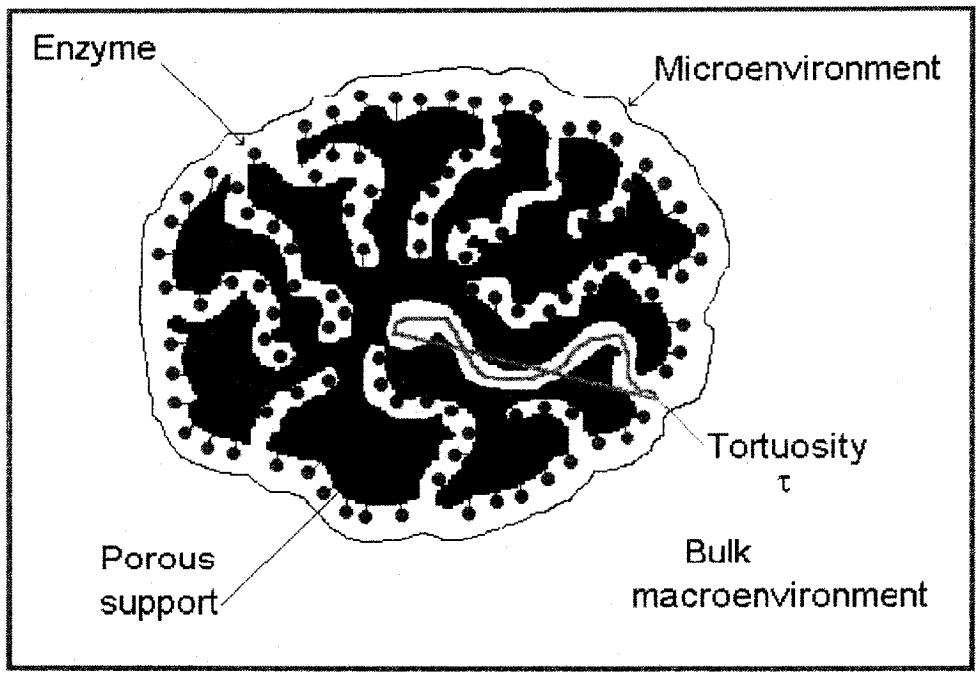


Fig.3.3: Diagram of immobilized macroenvironment

(http://emu0.emu.uct.ac.za/EMforBiologists/lecture2/lecture-2.htm, 2005-6-24)

(a) Assumed binding (b) Actual binding

Fig.3.4: Comparison between the assumed binding and actual binding

If the area occupied by one protein molecule is 1, and the molecules obey the monolayer binding, the calculated surface area is 5 (a). In the actual binding, because of the multilayer binding, the actual surface area is 4 (b), which is less than the calculated result.

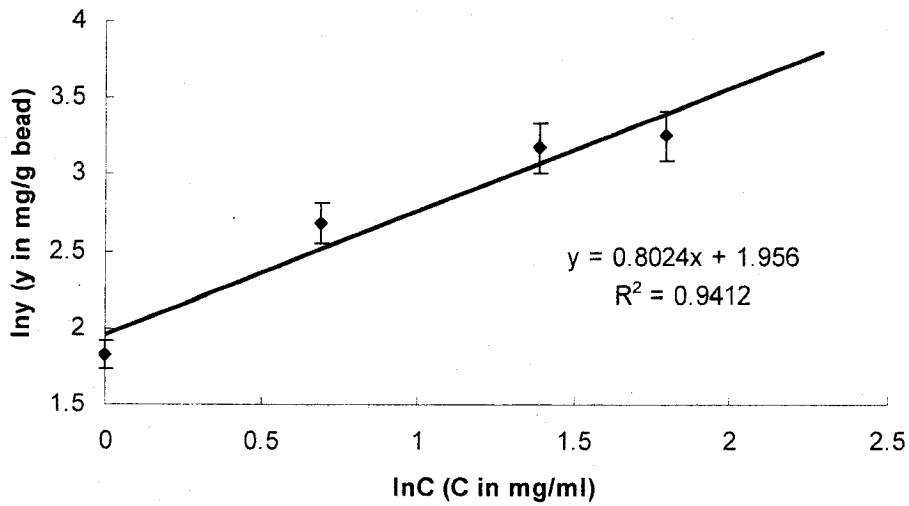


Fig. 3.5: Freundlich fitting curve of enzyme binding

Note: 1. C = trypsin solution concentration (mg/ml); y = amount of protein bound to the support material (mg/g bead); R^2 = measure of the fit of the regression line.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

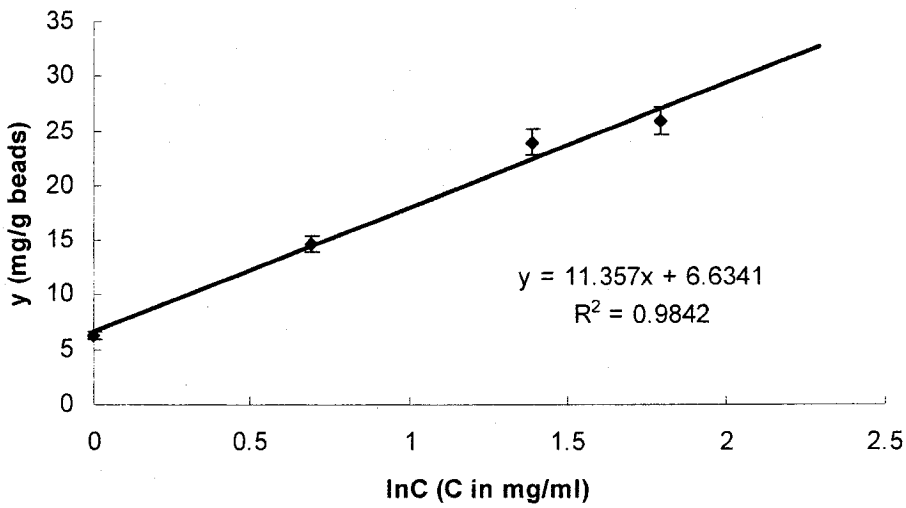


Fig. 3.6: Temkin fitting curve of enzyme binding

Note: 1. C = trypsin solution concentration (mg/ml); y = amount of protein bound to the support material (mg/g bead); R^2 = measure of the fit of the regression line.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

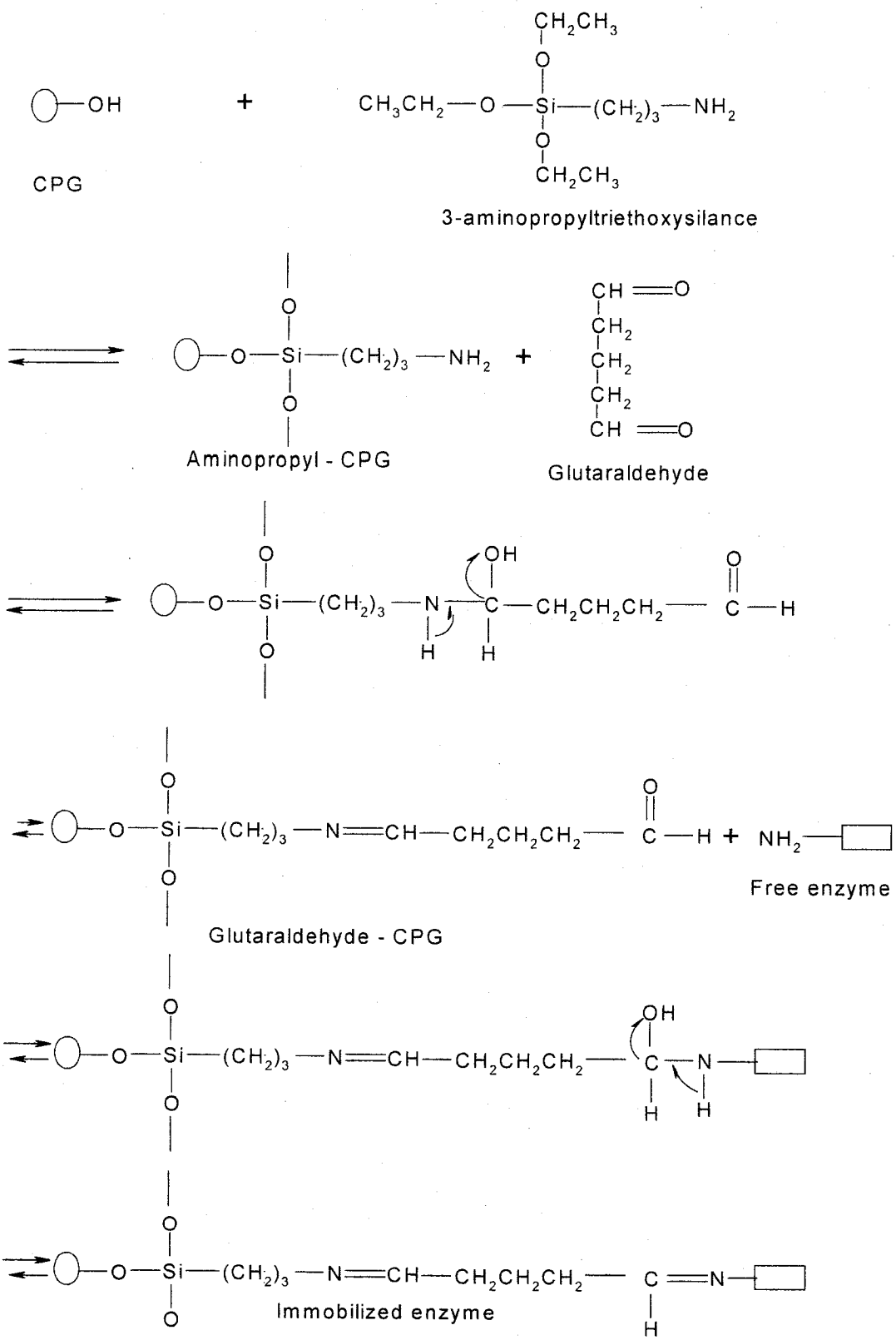


Fig. 3.7: Reaction pathway of enzyme immobilization with modified CPG

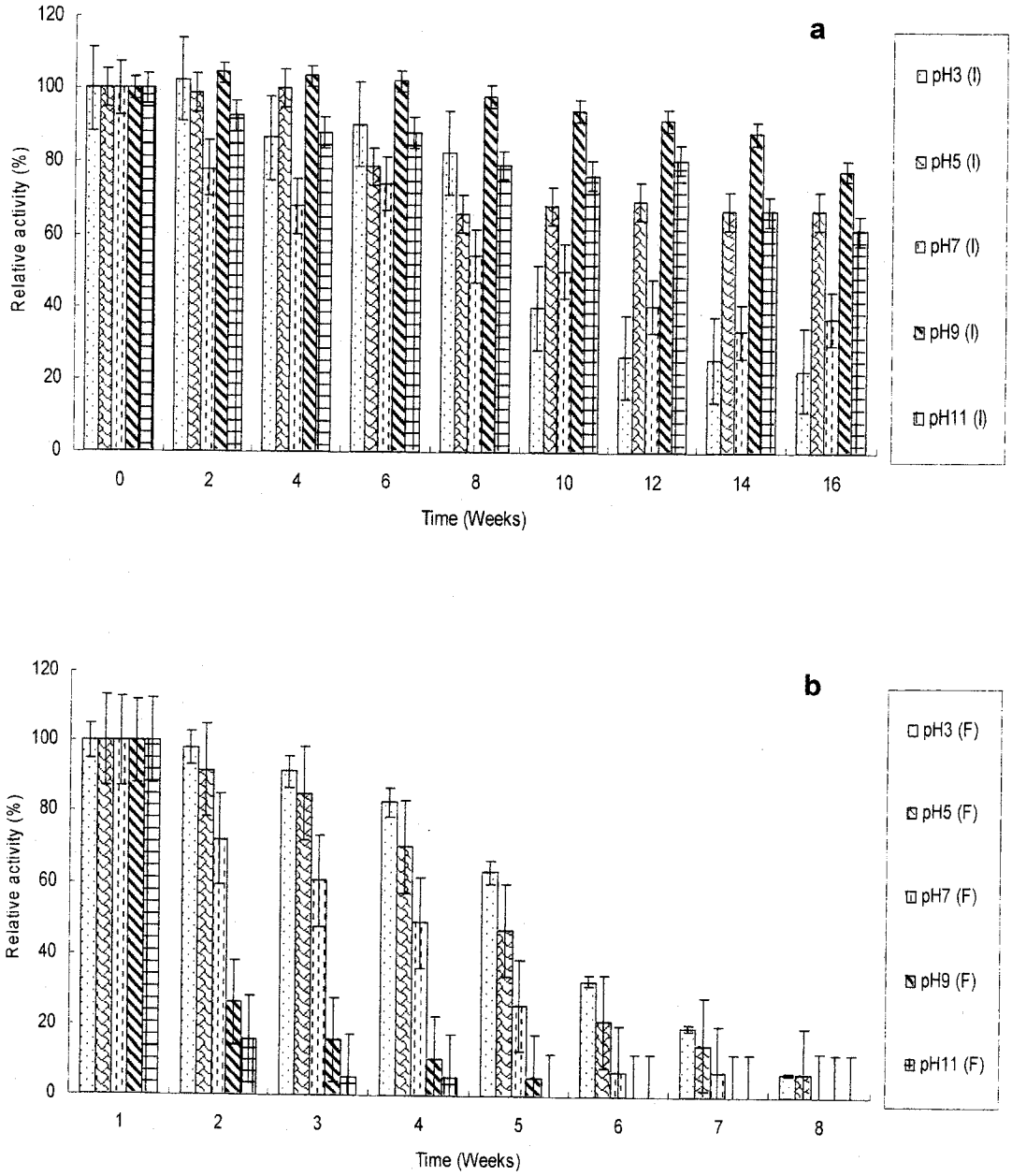


Fig.3.8: Storage stability of immobilized enzyme (a) and free enzyme (b) at different pHs

Note: Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

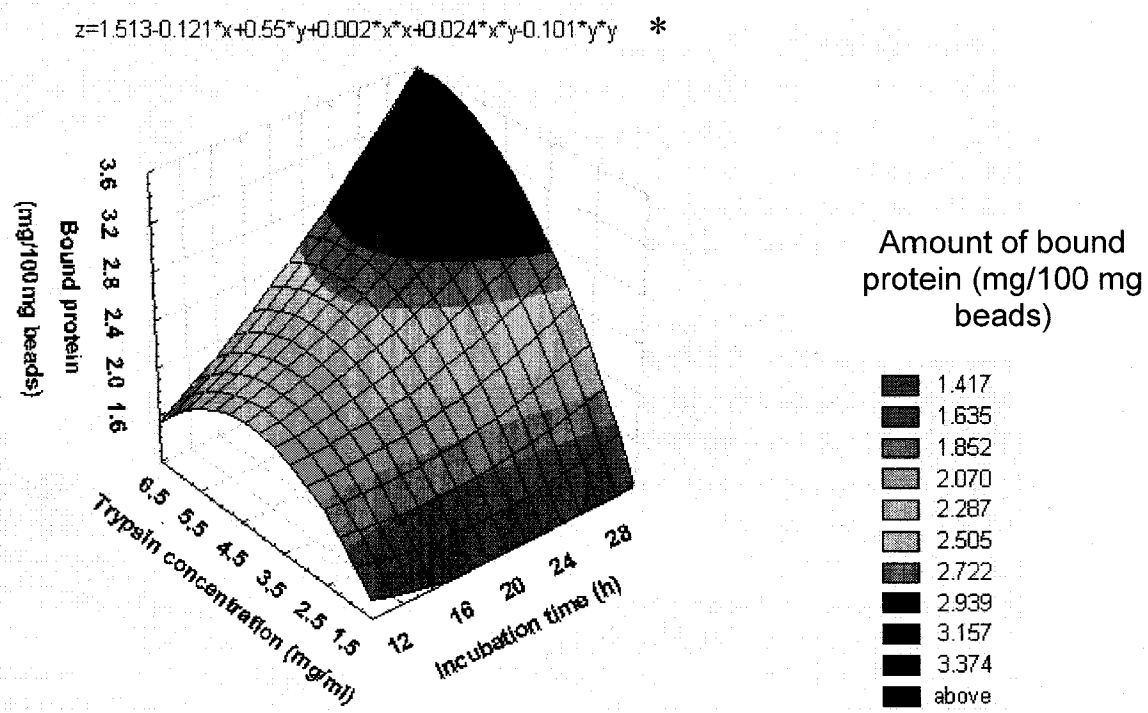


Fig.3.9: Effect of initial trypsin concentration and incubation time on the amount of protein bound onto CPG

* The equation describes how the amount of bound protein changes with the combined effects of trypsin concentration and incubation time, mathematically, where, x=trypsin concentration (mg/ml), y=incubation time (h), and z=bound protein (mg/100 mg beads). This equation is a second order regression of the bound protein as a function of trypsin concentration and incubation time.

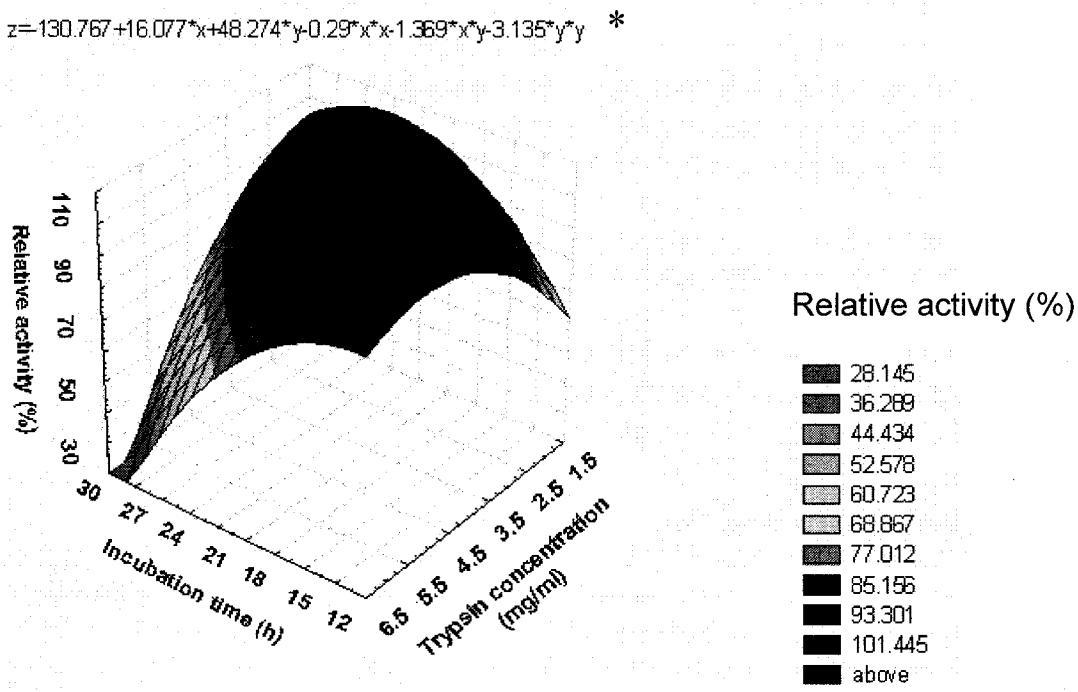


Fig.3.10: Effect of initial trypsin concentration and incubation time on the relative activity of immobilized trypsin

* The equation describes how the relative activity changes with the combined effects of trypsin concentration and incubation time, mathematically, where, x=incubation time (h), y=trypsin concentration (mg/ml), and z=relative activity (%). This equation is a second order regression of the relative activity as a function of trypsin concentration and incubation time.

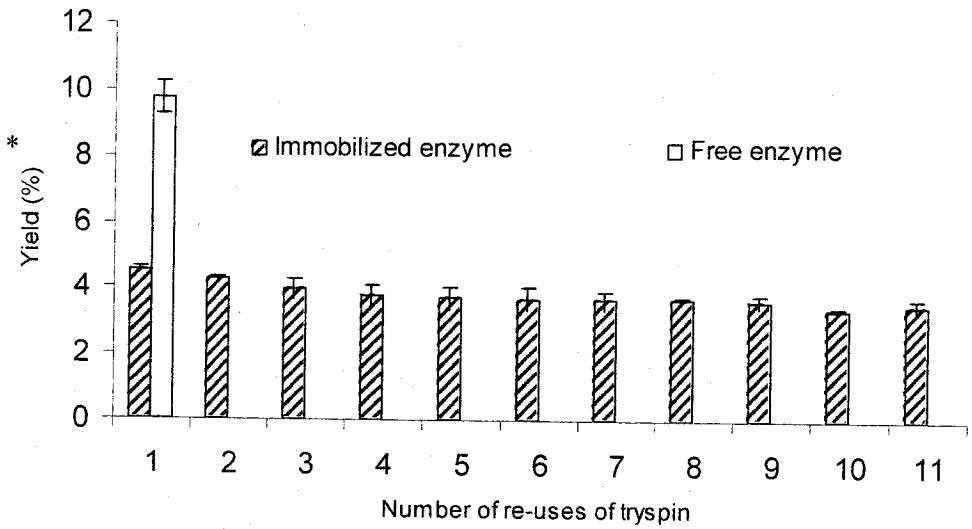


Fig. 3.11: Immobilized trypsin operational stability for extracting pigment from shrimp shell

* Yield was based on the dry weight of shrimp shell. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

CHAPTER IV

PROPERTIES OF IMMOBILIZED BOVINE TRYPSIN

Connecting Statement

In the previous chapter, bovine trypsin was immobilized onto controlled pore glass (CPG) beads, and the optimum conditions for immobilization, storage stability at various pHs, and operational stability (reusability) were evaluated. The results indicated that the obtained immobilized enzyme could keep its initial activity for several weeks and showed very high reusability. This chapter is about the properties of the immobilized bovine trypsin, such as, response to pH and temperature, sensitivity to inhibitors, kinetic properties, and effect of Ca^{2+} ions.

Note: This chapter constitutes the text of a paper to be submitted for publication as follows:

Li, D. and Simpson, B.K. Properties of immobilized trypsin on controlled pore glass (CPG) beads

Contribution of co-author(s): Simpson, B.K. (research / thesis supervisor) gave instructions to the candidate during research, provided financial support for the experiment, and edited the manuscript of this chapter.

Role of candidate: Candidate designed, conducted, analyzed and reported all the experiments presented.

ABSTRACT

Bovine trypsin was immobilized onto controlled pore glass (CPG) beads with glutaraldehyde as cross-linking reagent. The immobilized enzyme was characterized with respect to its kinetic properties, and for its response to pH, temperature and inhibitors in comparison with those of the free form of bovine trypsin. The immobilized trypsin showed lower binding affinity for its substrate, lower sensitivity to inhibitor and greater thermal stability, while the optimum pH and temperature were shifted to higher values compared to those of its free counterpart. The presence of Ca^{2+} ions increased enzyme loading onto the support material and stabilized the immobilized enzyme during storage, and enhanced catalysis by the immobilized enzyme.

Key words: Trypsin, enzyme immobilization, controlled pore glass (CPG), kinetic and physicochemical properties

INTRODUCTION

Enzyme immobilization is used to restrict the movement of enzyme molecules onto a stationary support material. This technique does not only fix enzyme molecules to make them reusable, but also has potential to modify the properties of enzymes, such as the stability and catalytic properties to enhance the feasibility of applications (Vidinha *et al.,* 2006; Yinghui *et al.,* 2002; Giacomini *et al.,* 2001). The main advantages of enzyme immobilization include reusability and ease of separation of the enzyme from substrate molecules. Thus, it could reduce the cost of processing and control the extent of hydrolysis of substrate (Wu *et al.,* 2005; Gomes *et al.*, 2004).

The methods used for immobilization include adsorption, covalent binding, encapsulation, entrapment and cross-linking, and may involve physical and / or chemical methods. Each method has its own advantages and disadvantages. The preferred method is application dependent (Bickerstaff, 1997a). For instance, glucose isomerase used in producing high - fructose corn syrup (HFCS) has been immobilized by several methods (Bhosale *et al.*, 1996): *e.g.,* adsorption onto an anion-exchange resin, entrapment involving filaments of cellulose acetate, adsorption onto specific SiO_2 particles followed by cross-linking with glutaraldehyde, or binding onto polythyleneimine–treated alumina via cross-linking with glutaraldehyde. Among these methods, covalent binding is widely used by most researchers (Limbut *et al.*, 2004; Kartal & Kilinc, 2006; Urek & Pazarlioglu, 2004). The main reason is that the obtained support – enzyme

complex is relatively stable (Vidinha *et al.,* 2006). Although this method is complicated and time-consuming, leakage of the enzyme molecules from support is unlikely, thus the immobilized enzyme could keep most of its initial activity for a relatively long time (Bickerstaff, 1997a; Scouten *et al.,* 1995).

Trypsin immobilization has wide application in the bioprocessing and food industries, such as, peptide mapping (Migneault *et al.,* 2004; Gobom *et al.,* 1997; Lippincott, *et al.,* 1997), affinity chromatography (Petro *et al.,* 1995), peptide synthesis (Sears & Clark, 1993), hydrolysis of casein (Kumar & Gupta, 1998; Malmsten, et al., 1999), and limited proteolysis of β-lactoglobulin (Chen *et al.,* 1994).

Various supports have been used in trypsin immobilization, such as, synthetic supports, acrylamide-based polymers (Bryjak & Kolarz, 1998), methacrylic acid-based polymers (Malmsten & Larsson, 2000), styrene-based polymers (Dautzenberg *et al.,* 1997), nylon (Isgrove *et al.,* 2001), agarose (Kim *et al.,* 2001), alginate (Taqieddin & Amiji, 2004), and chitin (Gomes *et al.,* 2004; Vaillant *et al.,* 2000; Ge *et al.,* 1996 a).

CPG was chosen as a support in this study, because it is an improvement over traditional soft gels, especially in processes that require high flow rates or high pressure (Limbut *et al.,* 2004). CPG is a porously structured material. It is immune to biological degradation and is compatible with almost all organic solvents and concentrated acids (except hydrofluoric acid) (Rosa *et al.,* 2002). As a solid support, CPG eliminates many of the problems experienced with gels

(Yamato *et al.*, 2004). CPG has a high surface area offering ligand coupling efficiency and high yield. The mechanical strength of CPG provides reproducible results with constant column parameters (Rosa *et al.*, 2002; Maquieira *et al.*, 1994). In addition, CPG is thermostable and autoclavable, so it is possible to use it in a sterilized environment (Chen *et al.*, 2004). CPG is available in different particle sizes, pore sizes and surface areas. The choice of which type of CPG to use for an application depends on the size of enzyme molecules, the applications, and handling properties (Millipore, 2006).

A major limitation of immobilized enzyme technology is its limited application with solid (insoluble particulate) substrates. For such substrates, it is hard to separate the immobilized enzyme from the products after the enzymatic reaction. In this study, large particle size of CPG was chosen in order to overcome this drawback of immobilized enzyme; so that the immobilized enzyme could be applied in extract pigment from shrimp shell in subsequent studies. By the different densities and solubilities of the support material and substrates / products, it is easy to separate the immobilized enzyme from the enzymatic reaction mixture.

Glutaraldehyde is a widely used cross-linking reagent to bind amino groups for immobilized enzyme preparations (Giacomini *et al.*, 2001; Juang *et al.*, 2001; Nouaimi *et al.*, 2001). Glutaraldehyde is not difficult to handle, if appropriate precautions are taken (Isgrove *et al.*, 2001). Moreover, glutaraldehyde acts as an arm of support material, so that the support holds the enzyme molecules firmly to prevent enzyme leakage from the support. Glutaraldehyde plays a role as spacer,

which could reduce the steric hindrance effect of the immobilized enzyme (Nouaimi *et al.*, 2001).

In this study, trypsin was immobilized onto CPG with large particle size, high surface area, and mechanical strength, so that the immobilized enzyme could be used in wide applications, such as with solid substrate, and extreme reaction conditions. The catalytic properties and kinetic parameters, as well as the stability of the immobilized trypsin were investigated. All these studies could provide useful information for using immobilized trypsin in other applications.

MATERIAL AND METHODS

Materials

Controlled pore glass beads (CPG2000A, surface area 11 m^2/g, bead mesh range 80/120) were purchased from CPG Inc, Lincoln Park, NJ. *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA), dimethyl sulfoxide (DMSO), 3-aminopropyltriethoxysilane, glutaraldehyde, bovine trypsin (type III), soybean trypsin inhibitor, glycine, EDTA, and nitric acid were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Sodium borate, sodium acetate, sodium chloride, calcium chloride, sodium hydroxide, hydrochloric acid, monobasic and dibasic potassium phosphate, and monobasic and dibasic sodium phosphate were purchased from Fisher Chemicals (Nepean, Ontario, Canada).

Preparation of controlled pore glass (CPG)

The procedure of Janolino & Swaisgood (1997) was used to clean the CPG beads. This was done by heating CPG beads in a boiling water bath with concentrated nitric acid (1:2 w/v) for 60 min. For glass beads that had been used previously, the beads were heated in a furnace at 600°C for 24 h before the boiling / incubation with concentrated nitric acid. The CPG beads were subsequently washed extensively in a large, coarse fritted-glass under suction with distilled water (approximately 1:20 w/v CPG to water), until the pH of the washings was neutral.

Synthesis of aminopropyl CPG

The method of Janolino & Swaisgood (1997) was used for the synthesis of aminopropyl CPG. A 10% (v/v) aqueous solution of 3-aminopropyltriethoxysilane was prepared and the pH was adjusted to 4.0 with 6 M HCl. Cleaned CPG was added to 3 vol of the 3-aminopropyltriethoxysilane solution. The mixture was degassed under vacuum to ensure that all of the entire pore volume was filled with the reagent and then incubated at 70°C for 3 h with occasional mixing. The excess reagent was decanted, and the wet CPG beads were placed in an oven at 100°C for 12 h for direct polymerization. The fine generated by mixing the beads was removed with a large volume of distilled water. The beads were washed with distilled water (about 10:1 v/w water to CPG) on a fritted-glass filter under suction, and dried for 12 h in an oven at 80°C.

Synthesis of glutaraldehyde-CPG

The synthesis of glutaraldehyde-CPG was based on the method of Sear and Clark (1993). Aminopropyl glass were washed with distilled water (about 1:100 w/v CPG to water) and suspended in 10 mM sodium phosphate buffer, pH 7.3, containing 10% glutaraldehyde (1:10 w/v CPG to buffer). The slurry was rotated end over end at room temperature (25°C) for 20 min, and then washed and filtered through a Millipore filter (0.45 µm pore size) with distilled water (about 1:500 w/v CPG to water).

Immobilization of enzyme

The procedure of enzyme immobilization was based on the method of Sear and Clark (1993). The activated glass beads were added to 10 mM sodium phosphate buffer, pH 7.3, (1:10 w/v CPG to buffer) containing 4 mg enzyme / ml buffer. The enzyme solution was recycled by pump (Micro tube pump MP-3, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and incubated with the glass beads for 21 h at 4° C. The glass beads were subsequently washed with 100 mM sodium borate / 1 M sodium chloride, pH 8.5, followed 100 mM sodium acetate / 1 M sodium chloride, pH 4.75 (1:250 w/v CPG to buffer). The washings were repeated for 4 times and finally CPG was washed with 10 mM sodium phosphate buffer, pH 7.3 (1:1000 w/v CPG to buffer). The damp beads were collected and lyophilized to dryness (in a LYPH-LOCK 12 freeze dry / shell freeze system, Labconco, Kansas City, Missouri).

The amount of bound protein was calculated as the difference between the concentration of protein in solution before and after immobilization. The protein concentrations of the enzyme solutions were determined by measuring their absorbances in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at both 260 nm and 280 nm using the formula below (Dunn, 1989):

$$protein\,(mg/ml) = 1.55\,A_{280} - 0.76\,A_{260} \qquad (4-1)$$

Activity determination of the immobilized trypsin

The activities of the free trypsins were determined by the method of Erlanger *et al.,* (1961) in terms of BAPNA units. For the immobilized trypsin, the method was modified as follows: 0.5g of dry immobilized trypsin in a test tube was heated in a water bath at 25°C for at least 15 min. About 5 ml of 1 mM BAPNA solution were then added to the immobilized trypsin. The test tube was shaken in a water-bath shaker (130 rpm, Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for exactly 10 min. The suspension was centrifuged at 12,000 g for 1 min at 25°C (in a Biofuge 13, Heraeus Instruments, Baxter Canlab, Mon-Royal, QC), and the absorbance of supernatant was measured with a spectrophotometer (Hitachi U 2000, Tokyo, Japan) at 410 nm. One BAPNA unit was defined as $\Delta A_{410nm/min}$ x 5 x 1000 / 8800, where 8800 is the extinction coefficient of *p*-nitroaniline and 5 is the total volume of reaction mixture.

Optimum pH of immobilized trypsin

The effect of pH in the range of 3 – 11 on the activity of both soluble and immobilized trypsin was measured with 1 mM BAPNA as substrate. The buffers were used as 10 mM citric acid – NaOH buffer for pH 3, pH 4 and pH 5; 10 mM potassium phosphate buffer for pH 6, pH 7, pH 8 and pH 8.5; 10 mM glycine – NaOH buffer for pH 9, pH 9.5 and pH 10; and 10 mM disodium hydrogen phosphate – NaOH buffer for pH 11. The substrate (1 mM BAPNA) was prepared with above buffers to obtain the selected pH values (pH 3 - 11). Five milliliters of 1 mM BAPNA with various pH and 0.5 g of trypsin immobilized beads were incubated in water bath at 25°C with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min. Then products were drained out from the trypsin immobilized beads, and the absorbance of product was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at room temperature (25°C).

Optimum Temperature of immobilized trypsin

The substrate (1 mM BAPNA) and the immobilized enzyme were incubated in a water bath at various temperatures (25°C – 80°C) for at least 15 min to equilibrate. Then 5 ml of 1 mM BAPNA and 1.00 g of trypsin immobilized beads were incubated in water bath at selected temperatures with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US). The time of the reaction was 10 min. Then supernatant were drained out from the trypsin immobilized beads, and the absorbance of product was measured at 410 nm in

an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan). The temperature range of study was from 25°C to 80°C.

Determination of thermostability of the immobilized enzyme

The thermal stability of the immobilized enzyme was determined by short-term and long-term incubation of immobilized enzyme at various temperature conditions.

Short-term incubation The immobilized enzymes and free enzymes were incubated at different temperatures (10 – 50°C) for 60 min. After incubation, the enzymes were taken out from the water bath and put into an ice bath to cool them down, then the residual activities of immobilized enzymes were checked as described in the text of "Activity determination of immobilized trypsin" at room temperature (25°C) with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate.

Long-term incubation This experiment was only carried out with the immobilized enzyme. The result of short-term incubation showed that immobilized enzyme had higher thermal stability, so the long-term incubation test was carried out with the immobilized enzyme. The immobilized enzymes were incubated at 30°C and 40°C for up to 7 days, and at 50°C, for 2 h, 4 h and 6 h (The preliminary results showed that after 6 h incubation at 50°C, the residual activity of immobilized enzyme was already below that of immobilized enzyme incubated at 30°C or 40°C for 7 days. That is why the experiment was not carried out for

longer incubation time at 50°C). After incubation, the enzymes were taken out from incubator and put into an ice bath to cool them down The residual enzyme activities were checked as described in the text of "Activity determination of immobilized trypsin" with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate.

Effect of soybean trypsin inhibitor on immobilized enzyme activity

Different concentrations of soybean trypsin inhibitor solutions (0 μM, 1 μM, 2 μM, 3 μM, 4 μM) were applied to determine the I_{50} values (the concentration of inhibitor necessary for a 50% inhibition). The inhibitor and substrate mixture was prepared by adding 100 μl of trypsin inhibitor solution with various concentrations (0 μM - 4 μM) to 5 ml of 1 mM BAPNA solution. 0.5 g of trypsin immobilized beads, substrate and inhibitor mixture were incubated in water bath at 25°C with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min. Then products were drained out from the trypsin immobilized beads, and the absorbance of product was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at room temperature (25°C). The assays for the free enzymes were carried out as follows. 200 μl of free enzyme solution (0.1% w/w) was added to the mixture of 2.8 ml of 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) and 50 μl of trypsin inhibitor solution with different concentrations (0 μM - 4 μM). The release of *p*-nitroaniline was measured at 410 nm at 25°C, using an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan).

Determination of kinetic parameters

For the determination of Michaelis-Menten kinetic parameters, different initial BAPNA concentrations (0.2 mM, 0.6 mM, 1.0 mM, 1.4 mM and 1.8 mM) were used. The reactions were carried out with constant amounts of the free enzyme (0.1% w/w) and immobilized enzyme (0.5 g) at 25°C.

The substrate (BAPNA) was prepared in 0.05 M Tris-HCl (pH 8.2, containing 0.02 M $CaCl_2$) to obtain the selected concentrations (0.2 mM – 1.8 mM). 0.5 g of trypsin immobilized beads and 5 ml of BAPNA with various concentrations were incubated in water bath at 25°C with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min. Then products were drained out from the trypsin immobilized beads, and the absorbance of product was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at room temperature (25°C). For the free enzyme, 200 µl of trypsin solution (0.1% w/w) was added to 2.8 ml of BAPNA solution with various concentrations in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02M $CaCl_2$) and the release of *p*-nitroaniline was measured at 410 nm at 25°C, using an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan).

Influence of Ca^{2+} ions

In order to examine the effect of Ca^{2+} ions on the immobilized enzyme activity, the immobilization process of the enzymes was carried out in the same buffer as

described for the other immobilized samples, by adding different concentrations of Ca^{2+} ions (as $CaCl_2$) were added.

The experiments on stability and kinetic study were carried out in the same way as described for the other samples by using BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2) as substrate.

RESULTS AND DISCUSSION

Optimum pH for immobilized and free trypsin

In this study, the immobilized trypsin showed the highest activity around pH 8.5 – 9, and optimum pH of the free enzyme is pH 8 (Fig. 4.1). Thus, the optimum pH of the immobilized trypsin shifted to higher pH compared to that of the free form. The possible cause of this phenomenon is that the negative charge was carried on the surface of the support. Binding of protons to the support material can result in an altered pH microenvironment around the support with consequent shift in pH optimum (1 – 2 pH units) of the immobilized enzyme (Bickerstaff, 1997a; Limbut *et al.*, 2004). Huang *et al.*, (1997) reported that the surface of support (succinamidopropyl-celite) carried a net positive charge and the immobilized trypsin shifted the optimum pH to lower value (pH 7.5) compared with free trypsin (pH 8). Kang *et al.*, (2005) indicated the optimum pH of immobilized trypsin onto a negative charged support (poly [(methyl methacrylate)-co-(ethyl acrylate)-co-(acrylic acid)] (P (MMA-EA-AA) latex particles) shifted to higher pH value (pH 8.5).

It is well known that the silica and silicate glass surfaces immersed in water acquire a negative surface charge, primarily through the dissociation of terminal silanol groups (Behrens & Grier, 2001).

$$SiOH \rightleftharpoons SiO^- + H^+ \qquad (4-2)$$

On the other hand, because the formation of double bonds of the modified CPG increased the negative charge density of the support, the modified CPG carried negative charge on the surface. Thereby, the negative charge on the surface of the support could alter the pH microenvironment around the active site of the enzyme. After the formation of the immobilized enzyme, the negatively charged support could increase the density of protons around the active site of the immobilized enzyme. Thus, the pH within the immobilized enzyme will be lower than that of the bulk solution. To compensate this effect, the optimum pH for the immobilized enzyme had to move to more alkaline range (Kang *et al.*, 2005).

Moreover, the pH profile of the immobilized trypsin was broader than that of the free form (Fig. 4.1). This indicates that the free enzyme is more sensitive to pH changes, and immobilization can preserve the enzyme activity over a wider range of pH. The possible reason is that the interactions between the enzyme and support could stabilize the conformation of active site of enzyme, thus, the immobilized enzyme could keep relatively higher activity at wider pH range. This improvement of property could enhance the feasibility of applying immobilized enzyme in industry.

Optimum temperature for immobilized and free trypsin

Optimum temperature is one of the important characters of the enzyme, where the enzyme shows the highest activity. However, if the temperature is too high, the enzyme could lose its activity because of the denaturation of the enzyme molecules. In Fig. 4.2, the immobilized enzyme showed higher optimum temperature (55°C) than that of the free enzyme (50°C). And the immobilized enzyme could keep relatively high activity at wider temperature range than that of the free form. For instance, the immobilized enzyme exhibited up to 90% of its maximum activity from 35°C to 60°C; in contrast, the free form exhibited the same level of activity only from 45°C to 50°C. This phenomenon indicates that immobilization technique could make the enzyme more robust than its free form. The formation of the covalent bonds because of the immobilization does not only limit the freedom of the enzyme molecules, but also changes their conformations, which lead to reduce their denaturation level. Moreover, because of the existence of steric hindrance of the immobilized enzyme, it needs more energy to make the substrate molecules gain access to the active site of the enzyme to cause the enzymatic reactions.

Thermal stability of immobilized and free trypsin

Fig. 4.3 and Fig. 4.4 show the short-term and long-term thermal stabilities of the enzymes, respectively. Figure 4.3 shows the results after incubation of the enzyme for 60 min at different temperatures from 10°C to 50°C, and indicates that the inactivation rate of the immobilized enzyme was much slower than that of the

free trypsin. For examples, from 10°C to 50°C, the immobilized trypsin and free trypsin retained 86% and 59% of their initial activities, respectively.

Figure 4.4 also shows the residual activity of the immobilized trypsin up to 7 days of incubation at different temperatures. At 30°C, the immobilized enzyme retained its activity at a relatively high level, *i.e.,* after 7 days, the immobilized enzyme still retained 84% of its initial activity. Even at a higher temperature (50°C), the residual activities of the immobilized enzymes after 2, 4 and 6 h incubation were 69%, 61%, and 38%, respectively. In contrast, for the free enzyme, Kang *et al.*, (2005) reported that the free trypsin retained less than 3% of its initial activity after 6 h of incubation at 55°C.

It is clear that the immobilization of trypsin onto controlled pore glass (CPG) enhanced its thermal stability remarkably. From the studies of long-term thermal stability, it could also be noticed that the thermal stability of the immobilized enzyme depends on both the temperature and the length of incubation period. Higher temperatures and / or longer incubation times could decrease the thermal stability of the immobilized enzyme. Hence, when immobilized enzymes are applied in industry, the temperature and duration of enzymatic reaction should be taken into consideration.

The increase in the thermal stability of the immobilized enzyme may be due to changes in the conformation and / or mechanical properties of enzyme caused by covalent binding between the enzyme molecules and the support material, as well as prevention of the autolysis of the enzyme.

Inhibition of immobilized enzyme by soybean trypsin inhibitor (SBTI)

Soybean trypsin inhibitor (SBTI) is one of the well known trypsin inhibitors. It comprises of plant proteins which have inhibitory activity against serine proteinases such as trypsin.

The residual activities of immobilized enzyme applied inhibitor are shown in Fig. 4.5, which indicates that the free trypsin was more sensitive to the inhibitor than the immobilized enzyme. With the same concentration of inhibitor solution, the free enzyme lost more activity than the immobilized enzyme. For instance, when the inhibitor concentration was 0.06 μM, the immobilized enzyme retained about 75% activity of the initial activity, while the free form of the enzyme retained only about 48% of the initial activity.

From a plot of V_o / V_i versus *[I]* (Fig. 4.6), the I_{50} of the immobilized and free enzymes were calculated as 0.1500 μM and 0.0543 μM, respectively. These data mean that to achieve 50% inhibition, the immobilized enzyme required more of the inhibitor (*i.e.,* 2.7 times that required by the free enzyme). Therefore, the immobilized enzyme is less sensitive to inhibition by SBTI than the free enzyme.

These phenomena may be explained using the scheme presented in Fig. 4.7, *i.e.*, after enzyme molecules had attached to the support, some of the active sites of the immobilized enzymes were oriented such that they faced away from the support (Type A); while for some of the enzyme molecules, their active sites were oriented to face to the support (Type B). Because the active sites of Type A

immobilized enzyme molecules were open to the media, the inhibitor molecules gained access to the active site easily to cause enzyme inhibition. So that the active sites of the Type A molecules were easily blocked by the inhibitor. On the other hand, for the Type B immobilized enzyme molecules, the active sites were shielded by the support partially or completely, so the inhibitor mclecules could not occupy the active sites of the enzyme to prevent binding and transformation of the substrate. When mixed with the substrate molecules, the size of substrate molecules (BAPNA, molecular weight: 138 Da) was much smaller than that of inhibitor (molecular weight: 20,100 Da) (Kolde & Ikenaka, 1973), thus the substrate molecules could easily diffuse and gain access to the active site and cause the enzymatic reaction. For the case of free enzyme molecules, the active sites were open to the media, which could bind the inhibitor readily, and obstruct substrate binding and transformation. From the above, it is obvious that the steric hindrance caused by the support material might protect the immobilized enzyme molecules from the inhibition. This feature of the immobilized enzyme could permit its use in certain applications such as modification of complex raw materials with naturally present inhibitors instead of the free form of the enzyme.

Influence of Ca^{2+} ions (as $CaCl_2$) to immobilized enzyme

The enzyme loading and residual activities of the immobilized enzymes in the presence or absence of Ca^{2+} ions were investigated (Table 4.1). As results shown in Table 4.1, the presence of Ca^{2+} ions could improve the loading of protein. For instance, in the system containing 0.01 M Ca^{2+} ions, the percentage of protein

bound to the support materials was increased to 83.63% versus 59.68% in the system without Ca^{2+} ions. Furthermore, the immobilized enzyme exhibited higher activities in the presence of Ca^{2+} than in the absence of Ca^{2+} ions. According to the data of residual activities of immobilized enzymes, when the system contained 0.01 M and 0.02 M Ca^{2+}, the activities of immobilized enzymes were 0.277 BAPNA units and 0.306 BAPNA units, respectively, both higher than the system without Ca^{2+} (0.265 BAPNA units). However, the concentrations of Ca^{2+} ions affect the protein loading and the residual activity of immobilized enzymes. For instance, at higher concentration of Ca^{2+} ions (*e.g.*, 0.04 M Ca^{2+}), the amount of protein bound as immobilized enzyme decreased to 40.63% with a corresponding reduced activity of 0.156 BAPNA units, which were lower than those in the absence of Ca^{2+} ions. It could be inferred that to the presence of high concentration Ca^{2+} ions has an influence on the electric charge of the protein molecules. The changes in protein charge may provoke some changes in the protein structures, which would affect the protein loading and enzyme activity exhibition (Ginalska *et al.,* 2000). The immobilized enzyme had the highest specific activity at the concentration level of 0.02 M Ca^{2+} (Table 4.1).

The storage stability of the immobilized trypsin in the presence of Ca^{2+} ions was also studied and the results are shown in Fig. 4.8 (a). The data indicated that Ca^{2+} ions affected the stabilities of the immobilized enzymes at the concentration levels of 0.01 M, 0.02 M and 0.04 M. The optimum storage stability was observed at a Ca^{2+} ion concentration of 0.02 M (Fig. 4.8 (a)). After four to six weeks of storage, the immobilized enzyme displayed higher activities compared with the

initial activity. The storage stabilities of free enzymes were also studied as reference (Fig. 4.8 (b)). The results indicated the free enzymes showed similar trends as the immobilized enzymes, but had less storage stability in the presence or absence of Ca^{2+} ions compared with the immobilized counterpart. It is well known that the presence of Ca^{2+} ions could improve the stability of free form of trypsin, which could be the possible reason for the increased storage stability of immobilized enzyme in the presence of Ca^{2+} ion.

The ability of Ca^{2+} to protect trypsin from self-digestion and subsequent stabilization of active enzyme is well established (Griffiths & Brecher, 1973; Sipos & Merkel, 1970; Cliffe & Grant, 1981). It is known that Ca^{2+} retards trypsin autolysis and promotes the activation of trypsinogen (Griffiths & Brecher, 1973). Addition of Ca^{2+} could cause a conformation change to a more compact structure and form a calcium-enzyme complex (Kotorman *et al.,* 2003; Yang *et al.*, 2002; Sipos & Merkel, 1970). Two Ca^{2+} binding sites have been proposed (Griffiths & Brecher, 1973). The primary site is very specific and results in a conformational change to a more compact structure. The second and less specific site is only found in trypsinogen and is believed to be on the group of four aspartyl residues on the peptide released during zymogen activation (Griffiths & Brecher, 1973). The secondary binding of Ca^{2+} to the group of carboxylates directs trypsin to the lysine-isoleucine bond which is cleaved in the process of trypsinogen activation (Delaage & Lazdunski, 1967). The formation of a calcium-trypsin complex could protect trypsin from a reversible inactive form, thus the active enzyme molecules are stabilized (Sipos & Merkel, 1970).

The primary site of calcium binding results in a conformational change to a more compact structure (Griffiths & Grecher, 1973). Subsequently, the enzyme molecules are stabilized at such structure, so that the immobilized enzyme obtained higher stability in the presence of Ca^{2+} ions (Fig 4.8). Moreover, such compact structure is also more suitable for enzyme molecules binding to the support materials – the same surface area could hold more enzyme molecules and with less accessing steric hindrance. So the amount of protein loading was increased in the presence of Ca^{2+} ions (Table 4.1). But high concentration of Ca^{2+} ions (*e.g.,* 0.04 M) could influence the electric charge of protein and change the protein structures. These changes could obstruct the protein loading and enzyme activity (Ginalska *et al.*, 2000).

The secondary site binding of Ca^{2+} ions is the result of trypsinogen activation, which could accelerate activation of trypsinogen (Griffiths & Brecher, 1973). This would be the possible reason for the increased activities of immobilized enzymes in the presence of Ca^{2+} ions after four or / and six week storage (Fig. 4.8 (a)).

Determination of kinetic parameters

The apparent Michaelis-Menten kinetic parameters (K_m' and V_{max}) for the free and immobilized trypsins were determined using the Lineweaver-Burk equation.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m'}{V_{max}}\frac{1}{S} \qquad (4 - 3)$$

where V is the initial or observed reaction rate, V_{max} is the maximum reaction rate,

S is the substrate concentration, and K_m' is the apparent Michaelis-Menten constant.

K_m' and V_{max} represent the binding affinity of enzyme to its substrate, and the maximum velocity of enzymatic reaction, respectively. From the slope and intercept of the plot of *1/V* versus *1/S* (Fig. 4.9), the kinetic constants for free and immobilized enzymes were obtained (Table 4.2).

The result showed K_m' of the immobilized enzyme was increased and V_{max} of the immobilized enzyme was decreased compared to its free counterpart. The K_m' value of the immobilized enzyme was increased 5 fold than that of the free form, which indicated that the affinity of the enzyme for its substrate is decreased. Similar results were reported by Huang *et al.*, (1997), Kang *et al.*, (2005), and Wu *et al.*, (2005). Huang *et al.*, (1997) reported that K_m' values of immobilized trypsin (onto aminopropyl-celite) and free enzyme were 3.60 mM and 0.15 mM. Kang *et al.*, (2005) reported the K_m' values of the immobilized trypsin (onto P(MMA-EA-AA) particles) and free trypsin were found to be 45.7 mg/ml and 30.0 mg/ml, and V_{max} to be 793.0 µg/min and 5467.5 µg/min, respectively. And the study of Wu *et al.*, (2005) presented that the K_m' values of immobilized trypsin (onto monodisperse PMMA microspheres) and free enzyme were 1.357 mM and 0.272 mM, and V_{max} values were 25 U/mg enzyme and 40 U/mg enzyme. The difference in K_m' of the free and immobilized trypsin may be attributed to the limited accessibility of the substrate to the active site of the immobilized enzyme, which could be caused by protein conformation changes induced by attachment to the support, steric

hindrance and diffusional effects (Ding & Qu, 2001).

On the other hand, V_{max} value of the immobilized enzyme was decreased 1.2 times than that of the free form. The effectiveness factor (*EF*) was introduced as a parameter to indicate the barrier effects of the immobilized enzyme on its activity. It was calculated by the following formula (Taqieddin & Amiji, 2004):

$$EF = V_{\max(immobilized\ enzyme)} / V_{\max(free\ enzyme)} \qquad (4-4)$$

If the *EF* value is equal to or greater than one, then there is no diffusion due to immobilization. If the *EF* is less than one, then the immobilization methods have an effect on the substrate and product diffusion (Taqieddin & Amiji, 2004).

In this study, *EF* value is 0.8, which is less than one. It means that there is a barrier effect of the immobilized enzyme. However, the value is close to one, which indicated that the barrier effect is not very strong, thus the mass transfer limitations were not significant. It is probably because the pore size of the support material is 2000 Å, which is much bigger than the size of enzyme molecule (38 Å), there is enough space for the substrate and product molecules to pass freely through the pores of the support material during catalysis.

The ratio of V_{max} / K_m' is another parameter to measure the efficiency of catalysis. If V_{max} / K_m' is large then the enzyme is very efficient. For this immobilized enzyme system, the ratio of V_{max} / K_m' of free enzyme is 9 times of that of immobilized enzyme. Because the free enzyme has higher affinity to its substrate

and higher activity compared to its immobilized form. However, the V_{max} / K_m' ratio of the free enzyme and immobilized enzyme were of the same order of magnitude. This means that the catalytic function of the enzyme was not impaired significantly by its immobilization.

The immobilized enzyme kinetics in the presence of Ca^{2+} ions was investigated as well, and the results were compared with that in the absence of Ca^{2+} ions. Linewaver-Burk plots of the immobilized enzyme in the presence or absence of Ca^{2+} are shown in Fig. 4.10, and the kinetic constants are summarized in Table 4.3. In the presence of Ca^{2+}, the immobilized enzyme had higher K_m', V_{max} *and* V_{max} / K_m' values compared with those in the absence of Ca^{2+} ions. The higher K_m' value of immobilized enzyme in the presence of Ca^{2+} ions means the immobilized enzyme had lower affinity to the substrate. However, the higher V_{max} value indicates that the immobilized enzymes had a higher capacity to transform the substrate to products, while the higher V_{max} / K_m' ratio suggests higher catalytic efficiency in the presence of Ca^{2+} ions. These findings are consistent with those of Kotorman *et al.,* (2003) who reported that Ca^{2+} ions could speed up the enzymatic reaction because of the formation of calcium-enzyme complex (Kotorman *et al.*, 2003).

In the reaction system containing Ca^{2+} ions, the calcium-trypsin complex was formed from a reversible inactive form, which slightly increases its proteolytic activity (Sipos & Merkel, 1970). Thus, the V_{max} value (Table 4.3) of the immobilized enzyme in the presence of Ca^{2+} is higher than that in the absence of

Ca^{2+} ions. Also, this compact structure could produce a barrier for the substrate molecules accessing to the active sites of enzyme molecules (Griffiths & Grecher, 1973), thus the affinity of immobilized enzyme to the substrate was lower and K_m' value was higher in the presence of Ca^{2+} ions than that in absence of Ca^{2+} ions (Table 4.3).

CONCLUSIONS

Immobilized trypsin had different properties compared to its free form. The differences were as follows: a higher K_m' value, higher optimum pH and temperature, and higher thermal stability and reduced sensitivity to inhibition by SBTI. Thus, immobilization does not only make the enzyme reusable, but also modifies some of its properties to alter its performance characteristics.

The differences in the kinetic properties of the immobilized versus the free enzyme suggest effects of steric hindrance on the affinity of enzyme for its substrate. Optimum pH was shifted to higher pH, which was caused by the electric charge microenvironment of the support. This phenomenon is an advantage for some application at higher pHs. The higher optimum temperature and thermal, inhibitory stability made it is possible to use the immobilized enzyme in extreme conditions.

The presence of Ca^{2+} ions could improve the protein loading to the support material, enhance the storage stability of the immobilized enzyme, and speed up the enzymatic reaction for immobilized enzyme.

Table 4.1: Amount of protein loading and enzyme activity of immobilized trypsin in the presence of Ca^{2+}

Condition	Protein added (mg/g CPG bead) A	Protein bound (mg/g CPG bead) B	Percentage of protein bound (B/A × 100 (%))	Activity of immobilized enzyme (BAPNA U/g CPG bead)	Specific activity (X 10^{-3} BAPNA U/mg bound protein)
Without Ca^{2+} ions	40	23.87	59.68	0.265	11.10
With 0.01 M Ca^{2+}	40	33.45	83.63	0.277	8.28
With 0.02 M Ca^{2+}	40	25.40	63.50	0.306	12.05
With 0.04 M Ca^{2+}	40	16.25	40.63	0.156	9.60

Note: Activity assays were carried out at 25° C with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2) as substrate. Specific activity was calculated by dividing activity of immobilized enzyme by the amount of bound protein.

Table 4.2: Kinetic parameters for immobilized and free trypsin

	K_m' (mM)	V_{max} (μmol/min)	V_{max} / K_m'
Free trypsin	0.55	178.57	322.58
Immobilized trypsin	3.97	142.86	35.97

Note: Substrate was BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl^2$) with concentrations of 0.2 mM, 0.6 mM, 1.0 mM, 1.4 mM and 1.8 mM. Reactions were carried out at 25° C and pH 8.2. Data are representative of three trials.

Table 4.3: Effect of Ca^{2+} on kinetic parameters of immobilized trypsin

	K_m' (mM)	V_{max} (μmol/min)	V_{max} / K_m'
Without Ca^{2+}	3.97	142.86	35.97
With Ca^{2+} (0.02M)	17.93	714.29	39.84

Note: Substrate was BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2) with concentrations of 0.2 mM, 0.6 mM, 1.0 mM, 1.4 mM and 1.8 mM, and reactions were carried out at 25° C and pH 8.2.

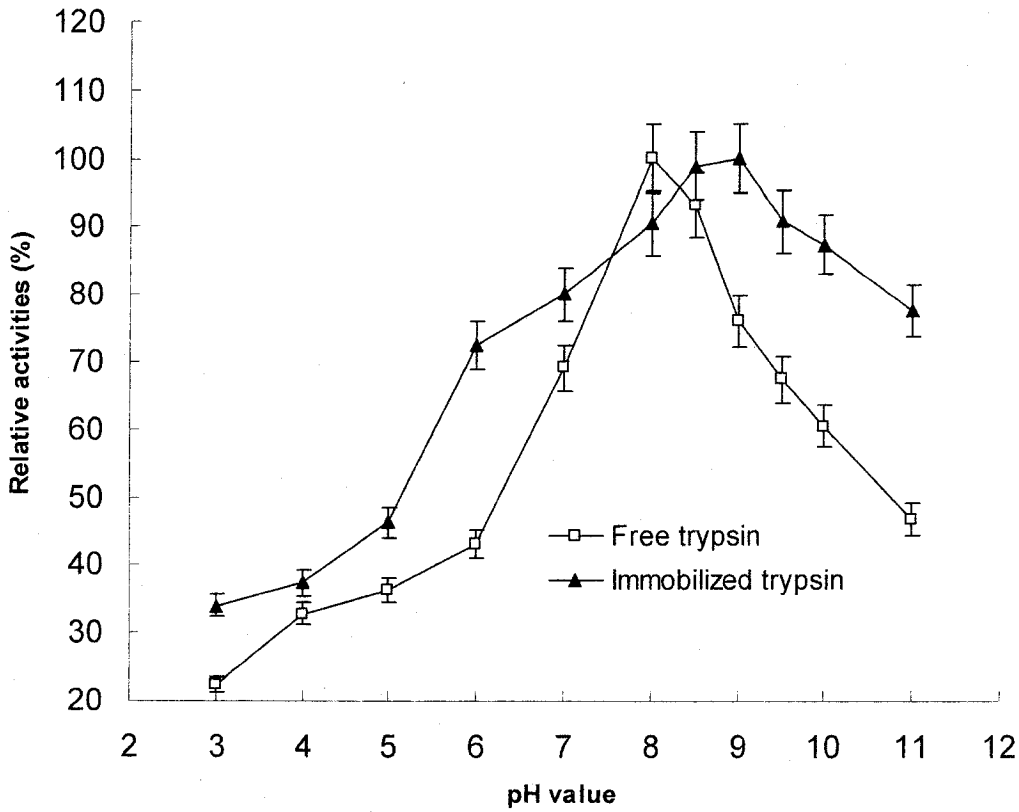


Fig. 4.1: Effect of pH on the activities of immobilized and free trypsin

Note: 1. Enzyme activities were measured at 25°C with 1 mM BAPNA as substrate.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

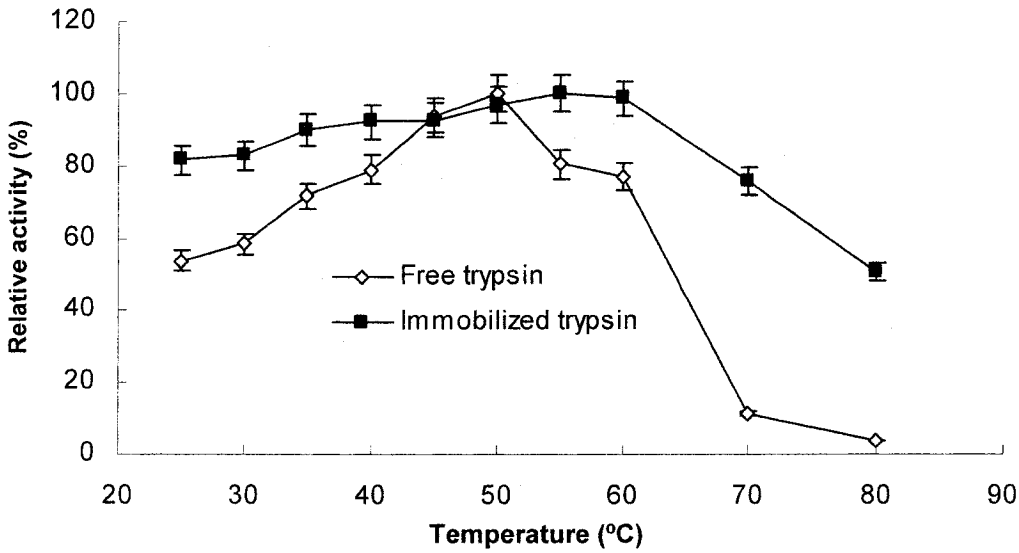


Fig. 4.2: Effect of temperature on the activities of immobilized trypsin

Note: 1. Substrate was 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) and reactions were carried out at selected temperatures as described in the text.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

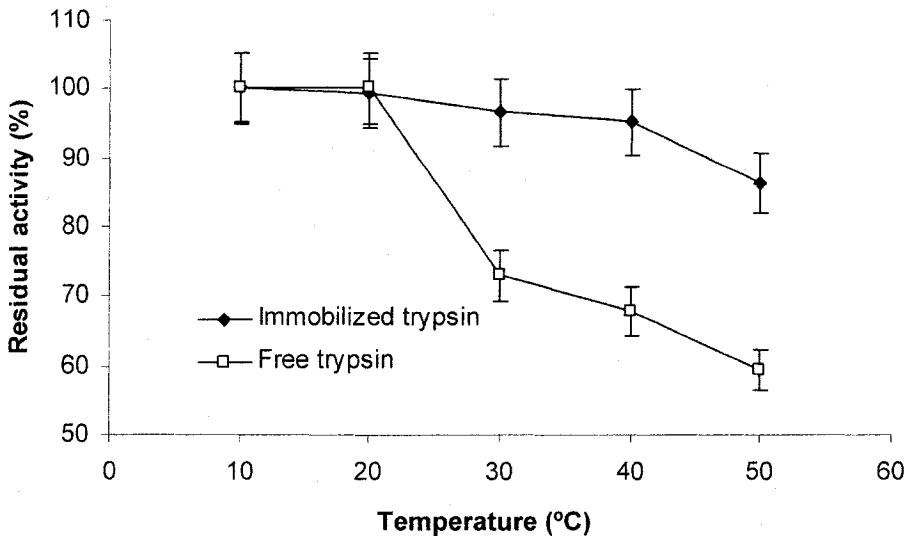


Fig. 4.3: Residual activity (%) for the immobilized and free trypsin after 60 min of incubation at different temperatures

Note: 1. Enzyme activities were measured at 25°C with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

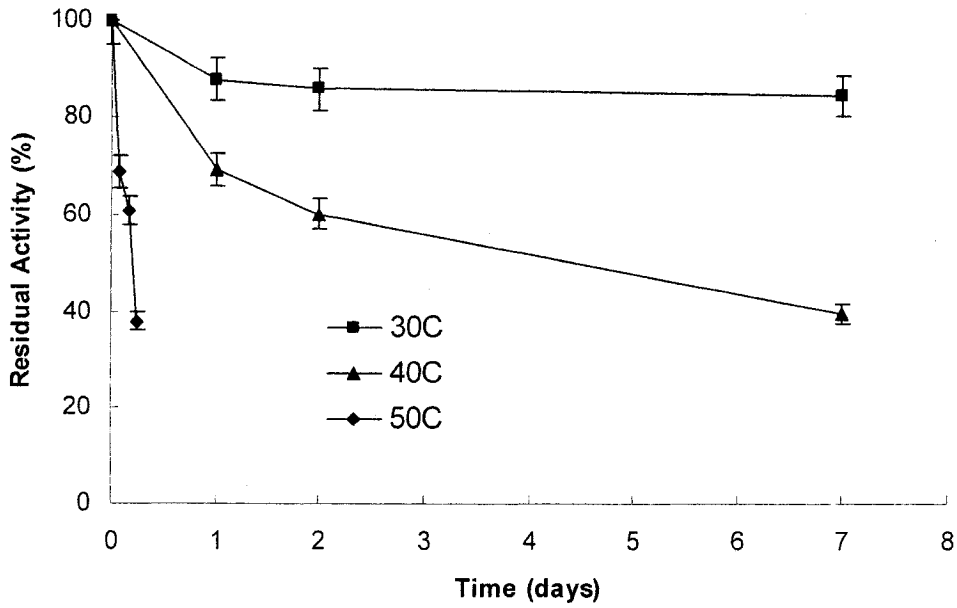


Fig. 4.4: Residual activity (%) as a function of time for the immobilized trypsin incubated at different temperatures

Note: 1. Enzyme activities were measured at 25°C with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

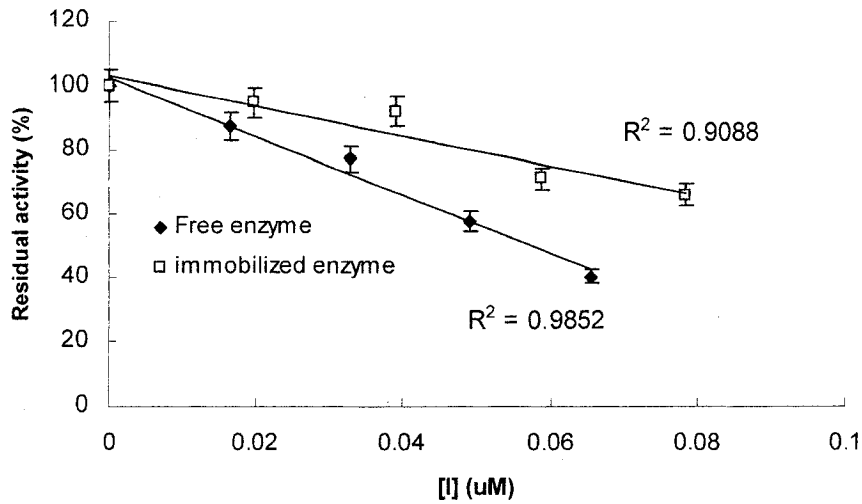


Fig 4.5: Residual activity (%) as a function of soybean trypsin inhibitor concentration for the immobilized and free trypsin

Note: 1. Enzyme activities were measured at 25°C with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

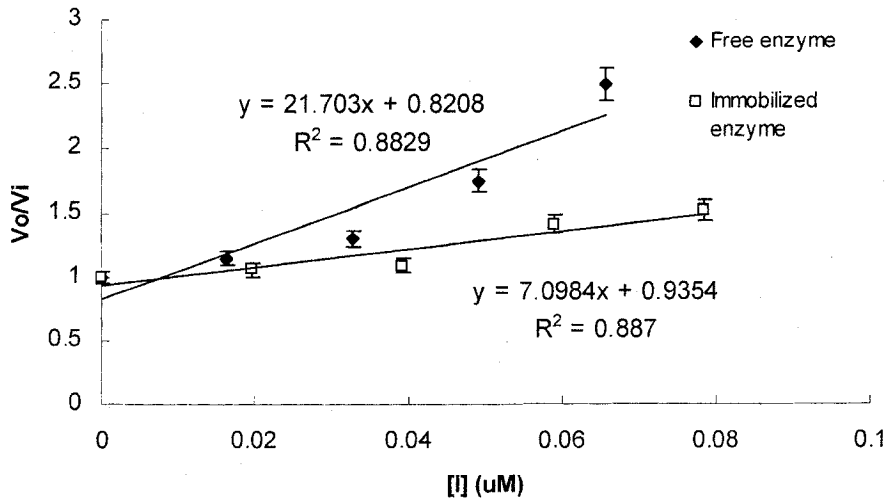


Fig. 4.6: Plot of *Vo/Vi* versus *[I]* for immobilized and free trypsins

Note: 1. Enzyme activities were measured at 25°C with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

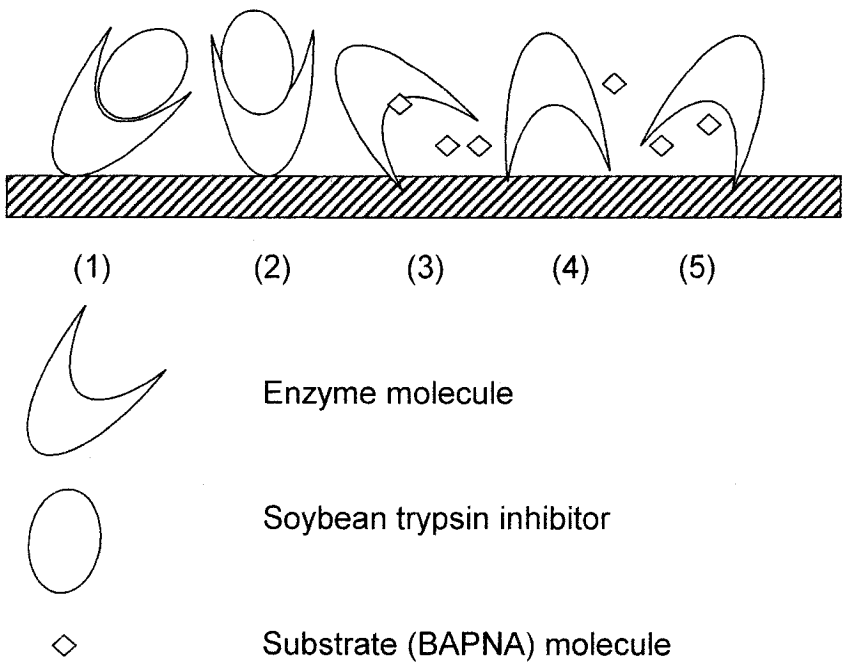


Fig. 4.7: Schematic of inhibition for immobilized trypsin.

(1)(2) Type A molecules, the active sites of the enzyme were blocked by the inhibitor; (3)(4)(5) Type B molecules, the active sites were facing to the support; (3)(5) there is space between the active sites of the enzyme molecules and support, thus the small size molecules such as substrate could gain access to the active site and cause the enzymatic reaction.

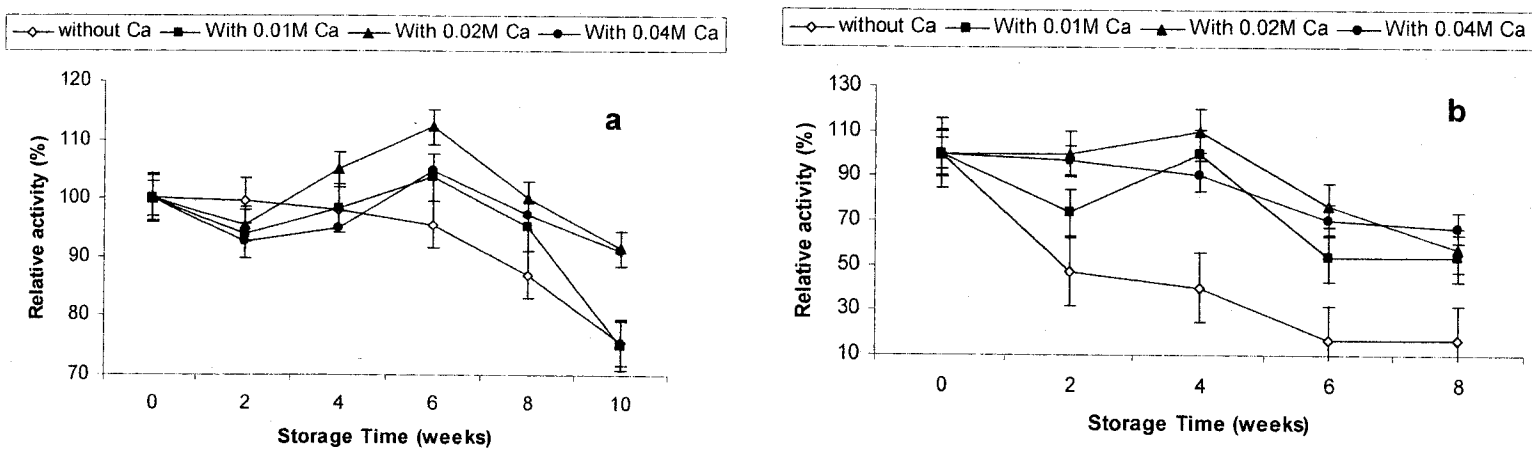


Fig. 4.8: Effects of Ca^{2+} on storage stability of immobilized enzyme (a) and free enzyme (b).

Note: 1. Enzyme activities were measured at 25°C with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2), and the immobilized enzyme was stored at 4°C during storage.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

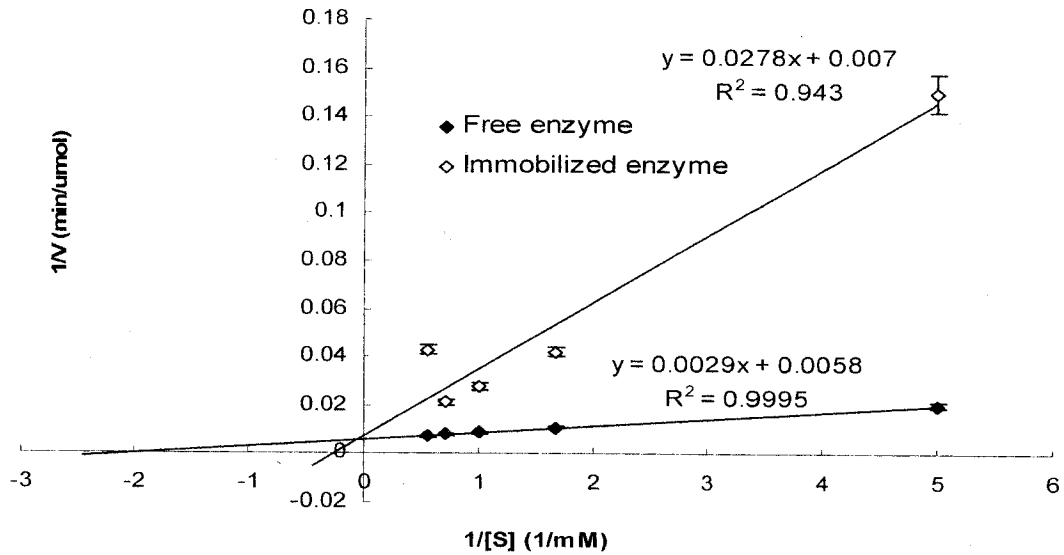


Fig. 4.9: Lineweaver-Burk plots of immobilized enzyme and free enzyme at 25°C

Note: 1. BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate, and concentrations were in the range of 0.2 mM – 1.8 mM. Trypsin activity was assayed at 25°C and pH 8.2. R^2 is "goodness of fit" of data to Lineweaver-Burk equation.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

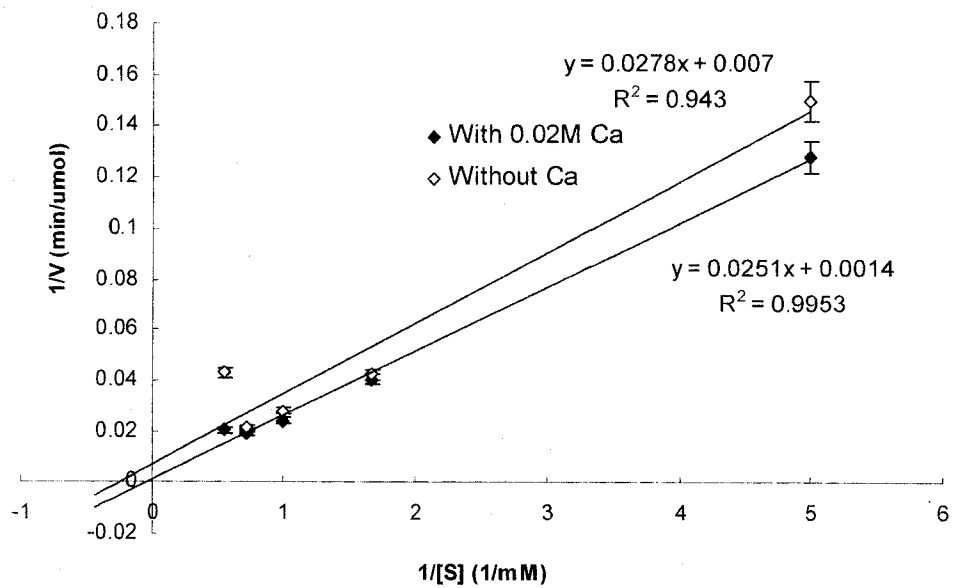


Fig. 4.10: Lineweaver-Burk plot of the immobilized enzyme with the presence of Ca^{2+} ions

Note: 1. Enzyme activities were measured at 25°C and substrate was BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2) with concentrations in the range of 0.2 mM – 1.8 mM. Data presented in figure is representative of two trials. R^2 is the measure of the fit of the regression line of Lineweaver-Burk equation.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

CHAPTER V

IMMOBILIZATION AND CHARACTERIZATION OF CUNNER FISH TRYPSIN

Connecting Statement

In the previous chapters, bovine trypsin immobilization and characterization were investigated. The results indicated that immobilized bovine trypsin had a higher K_m' value, higher pH and temperature optima, and was more thermal stable, but less susceptible to inhibition by SBTI compared with its free counterpart. Immobilization does not only make the enzyme reusable, but also alters part of its properties to improve its handling characteristics. In this chapter, trypsin was extracted from cunner fish tissue and immobilized onto controlled pore glass (CPG) beads. The properties of the immobilized fish trypsin were studied versus the immobilized bovine trypsin and / or free cunner fish trypsin, and the data obtained form the basis of this chapter.

Note: This chapter constitutes the text of a paper to be submitted for publication as follows:

Li, D., Simpson, B.K. and Squires, E.J. Immobilization and characterization of cunner fish trypsin

Contribution of co-author(s): Simpson, B.K. (research / thesis supervisor) gave instructions to the candidate during research, provided financial support for the experiment, and edited the manuscript of this chapter.

Squires, E.J. allowed me to carry out this research in his lab and provided facilities for experiment.

Role of candidate: Candidate designed, conducted, analyzed and reported all the experiments presented.

ABSTRACT

Cunner fish trypsin was extracted from pancreatic tissue, and immobilized onto controlled pore glass (CPG) beads using glutaraldehyde as cross-linking reagent. The influence of enzyme loading on degree of immobilization, immobilized enzyme activities and their storage stability at various pHs were determined. Maximum immobilization of the fish trypsin as well as optimum activity and stability were obtained at pH 9. The optimum pH and optimum temperature of the immobilized fish trypsin were shifted from pH 8.5 to pH 9, and from 45°C to 50°C, respectively, versus the free enzyme. The immobilized fish trypsin showed similar thermal stability at 30°C with the immobilized bovine trypsin, while at 40°C, immobilized fish trypsin displayed a thermal sensitivity similar to that of the free enzyme. The catalytic efficiencies (V_{max} / K_m') of immobilized fish trypsin were determined for both amidase and esterase reactions, and were found to be greater than those of the immobilized bovine trypsin. The operational stability of immobilized fish trypsin was studied by using it to facilitate extraction of carotenoprotein from shrimp shell. The immobilized fish trypsin retained about 75% of its initial hydrolytic capacity after 11 re-uses, and the yield obtained was over 20% higher than that obtained with immobilized bovine trypsin.

Key words: cunner fish trypsin, enzyme immobilization, controlled pore glass (CPG), properties

INTRODUCTION

Trypsin is a pancreatic protease, which acts on peptide bonds on the carboxyl side of lysine and arginine residues. Trypsin has a catalytic triad of serine, histidine and aspartate (Anonymous, 1972). The enzyme has several industrial applications, such as in medicine for both diagnostic and therapeutic purpose, peptide synthesis, characterization domain structures of proteins, and affinity purification of trypsin inhibitors (Bryjak & Kolarz, 1998). They are also used in the baking industry to improve the handling properties and texture of the products, for meat tenderization, recovery of proteins / peptides from bones, and hydrolysis of blood protein in the fish and meal industry (Simpson, 2000)

Digestive enzymes from cold adapted fish are a group of recently explored enzymes, which are more active catalysts at relatively low temperatures compared with similar enzymes from mammals, thermophilic organisms and plant sources (de Vecchi & Coppes, 1996). Low-temperature processing could provide various benefits, for example, lower thermal energy requirements, protection of substrates or products from degradation, minimization of side reactions, and prevention of the destruction of the heat-labile substances associated with raw material (de vecchi & Coppes, 1996).

Enzyme immobilization could make enzymes reusable, thus reducing processing cost, and enhance the thermal stability and handling properties of the enzyme. Bovine trypsin immobilization has been wildly investigated by many researchers (Sear & Clark, 1993; Ge *et al.*, 1996b; Chase & Yang, 1998; Isgrove *et al.*, 2001).

Both natural and synthetic polymers have been used as supports.

Trypsin from cold water fish exhibits different properties from bovine trypsin, such as different pH and temperature stability, kinetic properties differences, which are attributed to subtle structural difference between bovine trypsin molecules and fish trypsin molecules (Ciardiello, *et al.*, 2000; Clare, *et al.*, 2001). Based on the differences between the free forms of bovine and fish trypsins, it is speculated that the immobilized bovine trypsin may also be different from the immobilized fish trypsin. In this study, the conditions of immobilization and some properties of immobilized fish trypsin were investigated.

MATERIAL AND METHODS

Materials

Controlled pore glass beads (CPG2000A, surface area 11 m^2/g, bead mesh range 80/120) were purchased from CPG Inc, Lincoln Park, NJ. *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA), *p*-toluene-sulfonyl-*L*-arginine methyl ester (TAME), dimethyl sulfoxide (DMSO), 3-aminopropyltriethoxysilane, glutaraldehyde, bovine trypsin (type III), soybean trypsin inhibitor, ethylene-diamino-tetracetic acid (EDTA), Folin and Ciocalteu's phenol reagent, nitric acid, ammonium sulfate, cyanogen bromide (CNBr) activated Sepharose-4B, polyoxyethylene lauryl ether (Brij 35), acrylamide, bisacrylamide, ammonium persulfate (APF), sodium dodecyl sulfate (SDS), *NNN'N'*-tetramethyl ethylene diamine (TEMED), glycine, Coomasie brilliant blue (R-250) and 2-

mercaptoethanol were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Sodium borate, sodium acetate, sodium chloride, calcium chloride, sodium hydroxide, copper (II) sulfate, sodium carbonate, hydrochloric acid, monobasic / dibasic potassium phosphate, monobasic / dibasic sodium phosphate, Tris, acetone, trichloroacetic acid (TCA), acetic acid and propanol were purchased from Fisher Chemicals (Nepean, Ontario, Canada). Standard low molecular weight protein markers were purchased from Bio-Rad laboratories. Shrimp shell was obtained from Les Fruits de Mar (Matane, Quebec, Canada).

Biological specimens

Live cunner fish were caught by a fisherman at the coast of Miscou (New Brunswick, Canada) and transported live to the lab where they were held in tanks till needed.

Extraction and purification of trypsin from cunner fish

Trypsin fraction from cunner fish pancreas was prepared according to the method of Simpson and Haard (1985). The pancreatic tissue was taken from fish and rapidly frozen in liquid nitrogen, and then powdered in a waring blender. About 10 g of the tissue powder were mixed with 0.05 M Tris-HCl buffer (pH 7.8, containing 0.02 M $CaCl_2 \cdot 2H_2O$) at 4°C for 3 h (the ratio of tissue and buffer was 1:5 w/v). The homogenate was centrifuged at 3,000 g (in a J2-21 centrifuge, Beckman Coulter Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C to remove insoluble material and obtain the first supernatant (Sup 1). Sup 1 was

collected and was made up to 0.2% with Brij 35, and stirred at 4°C for 3 h, and then centrifuged at 10,000 g (in a J2-21 centrifuge, Beckman Coulter Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C to obtain the second supernatant (Sup 2). Sup 2 was fractionated with solid ammonium sulfate and the fraction precipitating between 40% and 60% saturation was collected by centrifugation at 6,000 g (in a J2-21 centrifuge, Beckman Coulter Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C. The $(NH_4)_2SO_4$ precipitate was dissolved in 20 ml of 0.05 M Tris-HCl buffer (pH 7.8, containing 0.02 M $CaCl_2$) and dialyzed for 12 h against three changes of 6 L of the Tris-HCl buffer to constitute the ammonium sulfate fraction. The dialysate was mixed with three times its volume of cold acetone (-20°C) and kept at -20°C for 3 h. The precipitate formed was collected by centrifugation at 6,000g (in a J2-21 centrifuge, Beckman Coulter Canada Inc, Ville Saint-Laurent, QC, Canada) at 4°C for 30 min, and then redissolved in 10 ml of the Tris-HCl to form the acetone fraction. The acetone fraction (10 ml) was pumped (Micro tube pump MP-3, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at a rate of 15 ml / h onto a soybean trypsin inhibitor (SBTI)-Sepharose 4B affinity chromatography column (that had been prepared as the procedure of Pharmacia Fine Chemicals; Anonymous, 1983). The column was thoroughly washed with elution buffer (0.05 M Tris-HCl buffer, pH 7.8, containing 0.02 M $CaCl_2$) to remove the unbound material after which the bound material was eluted with 5 mM HCl at a rate of 15 ml / h; and fractions of 4.8 ml / tube were collected. The fractions showing amidase activity were collected and adjusted to pH 7.8 with Tris-HCl buffer as affinity fraction and stored frozen at -20°C.

Enzyme activity assay (free form)

The amidase activity of trypsin fraction was estimated using the method of Erlanger *et al.* (1961) with *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA) as substrate. A 200 µl aliquot of an appropriately diluted enzyme extract was added to 2.8 ml of 1 mM BAPNA in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02M $CaCl_2$) and the release of *p*-nitroaniline was measured at 410 nm at 25°C, using an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan). One BAPNA unit of activity was defined as $\Delta A_{410nm/min}$ x 3 x 1000 / 8800, where 8800 is the extinction coefficient of *p*-nitroaniline and 3 is the total volume of reaction mixture.

Estimation of protein concentration

The protein concentrations of the enzyme fractions during extraction were determined by the method of Lowry *et al.*, (1951) as reported by Bickerstaff (1997b). To 0.6 ml of diluted protein sample, 2.5 ml of Lowry reagent mixture were added and the mixture was incubated at 25°C for 10 min. Then, 0.3 ml of diluted Folin reagent was added and vortexed immediately. The reaction color was developed for 30 min at 25°C. The absorbance of solution was read at 625 nm with an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan). A calibration graph of standard protein vs absorbance was prepared using a BSA solution of 1 mg/ml.

Protein determination: Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS – PAGE)

SDS-PAGE was used to determine the purity of the extracted trypsin and its molecular weight, using modified version of the method of Laemmli (1970). The polyacrylamide gel concentration of the resolving gel was 12.5%, and stacking gel was 3.5%. The gels were fixed with 12% TCA for 4 h, stained with 0.1% coomassie blue (R 250) for 12 h, and destained in 10% acetic acid and 20% propanol in water.

Protein standards were phosphorylase b (97.4 kDa), bovine serum albumine (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

Immobilization of cunner fish trypsin

The immobilized cunner fish trypsin was prepared based on the method of Janolino & Swaisgood (1997) and Sear & Clark (1993). Controlled pore glass (CPG) beads were washed with concentrated nitric acid. Previously used glass beads were heated at 600°C for 24 h prior to washing with concentrated nitric acid. The washed beads were derivatized as aminopropyl-CPG with 10% aqueous solution of 3-aminopropyltriethoxysilane, and the 10% glutaraldehyde was used to modify the beads to glutaraldehyde-CPG. The activated glass beads were then added to the enzyme solution, which was recycled and incubated for 21 h at 4°C. Next, the glass beads were washed with 100 mM sodium borate / 1

M sodium chloride, pH 8.5, and 100 mM sodium acetate / 1 M sodium chloride, pH 4.75 (1:250 w/v CPG to buffer). The washings were repeated for 4 times as above and finally the CPG beads were washed with 10 mM sodium phosphate, pH 7.3 (1:1000 w/v CPG to buffer). The damp beads were collected and lyophilized to dryness (in a LYPH-LOCK 12 freeze dry / shell freeze system, Labconco, Kansas City, Missouri).

Activity determination of the immobilized fish trypsin

The amidase activities of the immobilized trypsins were determined by the modified method of Erlanger *et al.*, (1961) in terms of BAPNA units. The method was modified as follows. 0.5 g of dry immobilized trypsin in a test tube was heated in a water bath at 25°C for 15 min. Next, 5.0 ml of 1 mM BAPNA solution were added to the immobilized trypsin. The test tube was shaken in a water-bath shaker (130 rpm, Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min. The solution was centrifuged at 12,000 g for 1 min at 25°C (in a Biofuge 13, Heraeus Instruments, Baxter Canlab, Mon-Royal, QC), and the absorbance of the supernatant was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan). One BAPNA unit of activity was defined as $\Delta A_{410nm/min}$ x 5 x 1000 / 8800, where 8800 is the extinction coefficient of *p*-nitroaniline and 5 is the total volume of reaction mixture.

The esterase activity of the immobilized trypsin was determined using a modified version of the method by Worthington Enzymes (Anonymous, 1972); *i.e.*, 0.5 g of the dry immobilized trypsin was mixed with 5.0 ml of 1mM TAME solution (in

0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$), and the mixture was incubated at 25°C for 10 min. The absorbance of product was measured at 247nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan). One TAME unit was defined as $\Delta A_{247nm/min} \times 5 \times 1000 / 540$, where 540 is the extinction coefficient of *p*-toluenesulfonyl-*L*-arginine at that wavelength, 5 is the total volume of reaction mixture.

Determination of bound protein

The protein concentration was determined by measuring the absorbance of the enzyme solutions at both 260 nm and 280 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) using the formula below (Dunn, 1989):

$$protein\,(mg/ml) = 1.55\,A_{280} - 0.76\,A_{260} \qquad (5\text{-}1)$$

The amount of bound protein was defined as the difference between the trypsin solution concentration before and after immobilization.

Effect of pH on fish trypsin immobilization and its storage stability at various pHs

The effect of pH on fish trypsin immobilization was studied in the pH range of 4 to 10 based on the methods of Vaillant *et al.*, (2000). Immobilized trypsins were prepared under different pH conditions. The buffers used were 10 mM citric acid –

NaOH buffer for pH 4 and pH 5; 10 mM potassium phosphate buffer for pH 6, pH 7 and pH 8; 10 mM glycine – NaOH buffer for pH 9 and pH 10. The immobilized trypsins were assessed for the amount of bound protein and the activity of immobilized trypsin. The storage stabilities of the immobilized fish trypsin at various pH conditions were verified by keeping the immobilized enzymes at 4°C in the buffer with different pHs and the residual activities were measured after two weeks intervals.

1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate to evaluate trypsin activity. 0.5 g of trypsin immobilized beads and 5 ml of 1 mM BAPNA were incubated in water bath at 25°C with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min. Then products were drained out from the trypsin immobilized beads, and the absorbance of product was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at room temperature (25°C).

Determination of optimum pH of the immobilized fish trypsin

The influence of pH on immobilized cunner trypsin activity was verified in the pH range of 3 to 11. The effect of pH on the activity of immobilized fish trypsin was measured with 1 mM BAPNA as substrate. The buffers used were 10 mM citric acid – NaOH buffer for pH 3, pH 4 and pH 5; 10 mM potassium phosphate buffer for pH 6, pH 7 and pH 8; 10 mM glycine – NaOH buffer for pH 9 and pH 10; and 10 mM disodium hydrogen phosphate – NaOH buffer for pH 11. The substrate (1 mM BAPNA) was prepared with above buffers to obtain the selected pH values

(pH 3 - 11). 5 ml of 1 mM BAPNA with various pH and 0.5 g of trypsin immobilized beads were incubated in water bath at 25°C with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min. Then products were drained out from the trypsin immobilized beads, and the absorbance of product was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at room temperature (25°C).

Determination of optimum temperature of immobilized fish trypsin

The effect of temperature on immobilized cunner trypsin activity was investigated for the temperature range of 10°C to 70°C. 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate. Substrate and immobilized enzyme were incubated in a water bath at various temperatures for at least 15 min to equilibrate. Then, 5 ml of 1 mM BAPNA and 0.5 g of immobilized fish trypsin beads were incubated in the water bath at selected temperatures with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US). The time of the reaction was kept for 10 min. Then products were drained out from the trypsin immobilized beads to terminate the reaction and the absorbance of product was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at room temperature (25°C).

Thermal stability of immobilized fish trypsin

Immobilized cunner trypsin was incubated at 30°C and 40°C up to 7 days. After incubation, the enzymes were taken out from incubator and put into an ice bath to cool them down. 0.5 g of immobilized trypsin beads in a test tube was heated in a water bath at 25°C for at least 15 min to equilibrate. About 5 ml of 1 mM BAPNA solution in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02 M $CaCl_2$) were then added to the immobilized trypsin. The test tube was shaken in a water-bath shaker (130 rpm, Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min at 25°C. The suspension was centrifuged at 12,000 g for 1 min at 25°C (Biofuge 13, Heraeus Instruments, Baxter Canlab, Mon-Royal, QC), and the absorbance of the supernatant was measured with an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at 410 nm. One BAPNA unit of activity was defined as $\Delta A_{410nm/min}$ x 5 x 1000 / 8800, where 8800 is the extinction coefficient of *p*-nitroaniline and 5 is the total volume of reaction mixture.

Determination of kinetic parameters of immobilized fish trypsin

For determination of Michaelis-Menten kinetic parameters, different initial BAPNA concentrations (0.05 mM, 0.35 mM, 0.65 mM, 0.95 mM, 1.25 mM) and TAME concentrations (0.25 mM, 0.50 mM, 0.75 mM, 1.00 mM, 1.25 mM) were used. BAPNA and TAME were prepared in 0.05 M Tris-HCl buffer (pH 8.2, containing 0.02 M $CaCl_2$), respectively. The reactions were carried out with constant amount of immobilized fish and bovine trypsin (0.5 g) at 25°C.

The substrate (BAPNA / TAME) was prepared in 0.05 M Tris-HCl (pH 8.2, containing 0.02 M $CaCl_2$) to obtain the selected concentrations. 0.5 g of fish / bovine trypsin immobilized beads and 5 ml of BAPNA / TAME with various concentrations were incubated in water bath at 25°C with shaking at 130 rpm (Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) for 10 min. Then products were drained out from the trypsin immobilized beads to terminate the enzymatic reaction, and the absorbance of product was measured at 410 nm in an UV / visible spectrophotometer (Hitachi U 2000, Tokyo, Japan) at room temperature (25°C).

Operational stability (Recovery of carotenoproteins with immobilized enzyme)

The operational stability of the immobilized trypsin was determined by the yields of extracted carotenoprotein, which is based on the method developed by Ya *et al.*, (1991). Before the reaction between shrimp shell and immobilized enzyme, the amount of protein bound to support material of immobilized enzyme was determined by the method described in "Determination of bound protein" part. Based on the amount of bound protein, the amount of shrimp shell was weighted as ratio of 1:1 (w/w) to the amount of bound protein. Then the grounded shrimp shell was incubated with immobilized trypsin in 0.1 M EDTA, pH 7.7 (1:3 w/v bound protein to EDTA buffer) at room temperature (25°C) for 6 h with continuous stirring. The mixture was filtered with cheese cloth, and the filtrate was collected and adjusted the pH to 4.5 with 6 M HCl, the filtrate was left at room temperature

(25°C) for 5 h. The mixture was centrifuged at 6,000g in a J2-21 centrifuge (Beckman Coulter Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C. The precipitate formed was collected and freeze-dried (LYPH-LOCK 12 freeze dry / shell freeze system, Labconco, Kansas City, Missouri). The operational stability was shown by the yield of extracted pigment from shrimp shell by using the same immobilized trypsin eleven times. In the control experiment, based on the amount of bound protein of immobilized enzyme, the same amount of free trypsin was used and the procedure was the same as the method described above. The free enzyme was used once and the yield of extracted carotenoprotein from shrimp shell was calculated based on the dry weight of shrimp shell.

RESULTS AND DISCUSSION

Trypsin purification

The results of trypsin purification from cunner pancreatic tissue are summarized in Table 5.1. The data indicate that the total protein and total activity decreased while the specific activity increased in the course of the purification.

The specific activity of the affinity fraction was 26 times greater than that of supernatant 1. The yield of the affinity fraction was 17% based on its total activity. The increase of the specific activity may be due to the activation of trypsinogen to trypsin (Pavlisko *et al.*, 1999). The total activity after the acetone step was 1.22 times greater than that of the ammonium sulfate fraction, which may be attributed

to the removal of naturally present trypsin inhibitors from the acetone fraction (Simpson and Haard, 1984 a).

Identity of protein fraction from affinity chromatography

The purity of the enzyme from affinity chromatography was examined by SDS-polyacrylamide gel electrophoresis (Fig. 5.1). The purified enzyme migrated as a single band in SDS-PAGE. From the plot of R_f vs Log M.W. (molecular weight), the molecular weight of the enzyme was obtained as 24,000 Da (Fig. 5.2). This result is similar to the values of trypsin from other sources of fish, such as, Greenland cod trypsin 23,500 Da (Simpson & Haard, 1984a, b), mullet trypsin 24,000 Da (Guizani *et al.*, 1991, Pavlisko *et al.*, 1999).

Effect of pH on fish trypsin immobilization

The amount of enzyme protein bound to the support material, the activities of the immobilized enzymes and its storage stability at different pHs are summarized in Table 5.2 and Fig. 5.3. Table 5.2 indicates that at pH 6 and pH 9, the immobilized fish trypsin showed higher activities than at other pHs, and the highest amount of bound protein was obtained at pH 9. The storage stability study (Fig. 5.3) shows that at pH 9, the immobilized enzyme was stable and retained its initial activity for up to 8 weeks. After 8 weeks of storage, the immobilized fish trypsin retained 80% of its initial activity.

The reaction pathway of the immobilized fish trypsin is similar with that of the

immobilized bovine trypsin. Firstly, - OH groups on the surface of CPG was modified by 3-aminopropyltriethoxysilance, to produce the aminopropyl-CPG. And then the modified beads reacted with glutaraldehyde to attach the $-\overset{O}{\overset{\|}{C}}-H$ to the beads. Finally, the activated beads reacted with the $-NH_2$ on the surface of the enzyme molecules by the functional group $-\overset{O}{\overset{\|}{C}}-H$. The possible reaction principle is the Schiff base reaction.

In this Schiff base reaction, the reaction activity depends on the concentration of $>C=O$ and the $-NH_2$ in the solution. According to this reason, the solution should be basic or neutral to prevent formation of $\overset{+}{R}NH_3$. On the other hand, during reaction, there is a proton transfer in acetal formation, which is promoted by acid. Thus, the pH of the solution should be acid enough to facilitate the proton transfers. The pH of Schiff base reaction media should be in an intermediate pH at which the rate of reaction is a maximum (Brown, 1975). Zabinski and Toney (2001) reported that at more alkaline pH (*i.e.*, 9 – 10), Schiff base formation occurs rapidly and with high efficiency, resulting in greater incorporation of enzyme, and the stability of Schiff base decreases with decreasing pH. So, these could explain the reason why at pH 9, the immobilized enzyme showed high activity and with more protein bound to support.

At pH 9, the immobilized fish trypsin could obtain the highest amount of protein bound to support, which could reduce the activity loss of the immobilized enzyme

due to the leakage of protein from support material. At that pH, during storage, the activity of the immobilized enzyme kept its highest activity up to 8 weeks compared to the immobilized enzymes at other pHs.

Optimum pH of immobilized fish trypsin

The data on optimum pH studies for the immobilized fish trypsin are shown in Fig. 5.4. Immobilized enzymes usually display a broad optimum pH range, and showed higher activities at extreme pH values compared to its free form (Huang *et al.*, 1997). In our previous studies on the immobilized bovine trypsin, the result also showed a similar trend. However, the immobilized fish trypsin did not show wider optimum pH range compared to its soluble counterpart (Fig. 5.4).

The optimum pH of the immobilized fish trypsin is pH 9, which is similar with the data of bovine trypsin bound to CPG. The optimum pH of the immobilized enzyme mainly depends on the charge distribution on the surface of the support (Limbut *et al.*, 2004).

In this study, controlled pore glass (CPG) was used as support with silicate glass surfaces. A negative charged surface will be formed when the silicate glass surface immersed in water acquire (Behrens & Grier, 2001).

$$SiOH \rightleftharpoons SiO^- + H^+ \qquad (5-2)$$

Moreover, because of the formation of double bonds during support modification,

it could increase the negative charged density of the support. Thus, the support carried a net negative charge on its surface. The negative charge affected the optimum pH of the immobilized enzyme shifted to higher pH value.

The same enzyme immobilized onto different support material could show different pH optimum because of the net charge of the support material. For instance, bovine trypsin was immobilized to succinamidopropyl – celite (SAPC) with net positive charge on the surface (Huang *et al.*, 1997). Such immobilized bovine trypsin, the optimum pH was pH 7.5, which was lower than the optimum pH of its free form (pH 8, Anonymous, 1972). However, the same enzyme was immobilized onto poly[(methyl methacrylate)-co-(ethyl acrylate)-co-(acrylic acid)] latex particles carrying a negative charge, which affects the optimum pH of immobilized bovine trypsin to higher value (pH 8.5) versus the free enzyme (Kang, *et al.*, 2005).

On the other hand, the net charge on the surface of the enzyme does not seem to affect optimum pH of the immobilized enzyme. In this study, bovine trypsin and cunner fish trypsin were immobilized onto the same support material CPG. From the isoelectric point of bovine trypsin ($PI_{trypsinogen}$ = 9.3; $PI_{trypsin}$ = 10.1) (Anonymous, 1972) and cunner fish trypsin (PI = 5.70) (Macouzet, 2004), it indicated that bovine trypsin carries net positive charge, and net charge is +6 (Genicot *et al.*, 1996); cunner fish trypsin carries net negative charge. The optimum pH for both of them was pH 9.0. Thus, it could be assumed that the optimum pH of the immobilized enzyme is affected by the net charge of the

support materials, but not the net charge of the enzyme. From this point of view, different support with different net charge could be selected, and subsequently change the optimum pH of immobilized enzyme, in order to suit different applications. Hence, the same enzyme will be applied in much wider pH range by selecting different support material. However, generally the optimum pH of the immobilized enzyme should be determined by experiment.

Optimum temperature of immobilized fish trypsin

The data for the optimum temperature study of the immobilized fish trypsin are presented in Fig. 5.5. Similar results were obtained by Huang *et al.*, (1997), Bryjak & Kolarz (1998), Kumar & Gupta (1998) and Kang *et al.*, (2005). Immobilization increased the optimum temperature of fish trypsin from 45°C to 50°C. It may be explained that the covalent bonds between the enzyme molecules and support material stabilized the conformation of the enzyme molecules. Thus, the active site of the immobilized enzyme was more stable, even under higher temperature, compared to the free form. Kang *et al.*, (2005) also reported that immobilized enzymes have higher optimum temperature than its free counterpart usually.

Thermal stability of immobilized fish trypsin

The data for the thermal stability of the immobilized fish enzyme are summarized in Fig. 5.6 (30°C) and Fig. 5.7 (40°C). The results were compared with those of the immobilized bovine trypsin. Fig. 5.6 shows similar thermal stability at 30°C for

both the immobilized bovine and fish trypsins. They were quite stable even after 7 days storage at 30°C, retaining about 81% and 84% initial activities for immobilized fish trypsin and bovine trypsin, respectively. However, for short time storage (*e.g.,* 1 day), the immobilized fish trypsin exhibited higher stability than that of the immobilized bovine trypsin (Fig. 5.6). In general, fish trypsin is quite stable at relatively low temperature compared with the similar enzyme from mammals. At 30°C for 1 day incubation, the immobilized fish trypsin also showed relatively high thermal stability compared with immobilized bovine trypsin. But for longer incubation, the immobilized fish trypsin showed its thermosensitivity compared to its bovine counterpart.

However, at higher temperature, for instance, 40°C, the immobilized fish trypsin was more heat labile compared to the immobilized bovine trypsin (Fig. 5.7). Genicot *et al.*, (1996) reported that trypsin from Antarctic fish was rapidly inactivated at temperatures higher than 30°C. The immobilized fish trypsin showed the similar trend with the free form in this study. Ahsan *et al.,* (2001), explained that anchovy trypsin lacks Tyr-151 in the substrate binding pocket, resulting in an overall decrease in the number of salt bridges and hydrophobicity in comparison with bovine trypsin, which might contribute to their thermosensitivity. From the amino acid sequence of cunner fish (Macouzet, 2004), the catalytic triad includes His-57, Asp-102, and Ser-195, and the obligatory Asp-189, also lacks Tyr-151. So a similar explanation could be obtained from the structural features. Although, immobilization could enhance the stability of the fish enzyme, the thermosensitivity of the fish enzyme also affected the thermal

stability of the immobilized form under higher temperature.

Kinetic properties of immobilized fish trypsin

By using Lineweaver–Burk plot (Figs. 5.8 & 5.9), the kinetic properties of the immobilized fish trypsin were obtained (Table 5.3), and the results were compared to those of immobilized bovine trypsin. The substrates used were *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA) and *p*-toluene-sulfonyl-*L*-arginine methyl ester (TAME), respectively, in order to determine the catalytic capacity for amidase and esterase reaction.

In this study, the catalytic efficiencies (V_{max} / K_m') for the amidase and esterase reaction were greater for immobilized fish trypsin than those for immobilized bovine trypsin (Table 5.3). It indicated that the immobilized fish trypsin had more catalytic capacity on both amide and ester than the immobilized bovine trypsin.

For the free form, Genicot *et al.,* (1996) compared the structural features of the binding pockets of the bovine and fish (Antarctic fish) trypsins, which revealed a deletion of residue Tyr-151 in Antarctic fish enzyme. In bovine trypsin, residue Gln-192 is located at the entrance of the binding pocket and there was only 3.2 Å in distance. So the fish enzyme residue Gln-192 has a lot of freedom compared to bovine trypsin. On the other hand, the next residue of Gln-192 is proline in bovine trypsin, which is substituted by another smaller size of residue glycine in fish trypsin (Genicot *et al.*, 1996). Thus, the structural characteristics of the binding pocket of the fish enzyme are helpful for the substrate binding readily to

the active site of the enzyme, which could explain the reason why fish enzyme has a high catalytic efficiency at low and moderate temperature. Macouzet (2004) revealed the amino acid sequence of cunner fish trypsin, and the identities of nucleotides and amino acid with Antarctic fish was 90% and 86%, respectively, and the main residue mentioned above were included. Therefore, the above theory could also be applied to cunner fish trypsin with high catalytic efficiency.

Operational stability of the immobilized fish trypsin

The operational stability of the immobilized enzyme was estimated by extracting carotenoproteins from shrimp shell, the results were compared to that of the immobilized bovine trypsin (Fig.5.10). This method used for extracting pigment was carried out at room temperature (25°C). It aimed at using lesser investment to obtain higher yield of pigment. The variations in the yields may be due to loss of activity from the immobilized enzyme and / or fluctuations in temperature.

The results showed that the yield remained quite high even after 11 re-uses of the immobilized enzyme, and either the free form or the immobilized form of fish trypsin showed higher catalytic capacity than that of bovine trypsin. After 11 re-uses, the immobilized fish enzyme retained 75% of its initial activity. On the average, the immobilized fish trypsin had 20% of catalytic capacity higher than that of immobilized bovine trypsin. Therefore, immobilization does not only make the enzyme reusable, but also keeps the characteristics of its free form.

CONCLUSIONS

The cunner fish trypsin was successfully extracted and immobilized onto controlled pore glass, and it could be applied in carotenoprotein extraction from shrimp shell.

The immobilized fish trypsin obtained its highest protein load at pH 9, and at that pH the immobilized enzyme showed high activity and kept its activity for long time up to 8 weeks. On the other hand, the immobilization process changed the characteristics of the enzyme, for example, the optimum pH and temperature were shifted to higher values compared to its soluble counterpart. The immobilized fish trypsin showed higher amidase and esterase activity than that of the immobilized bovine trypsin. Moreover, the immobilized fish trypsin was applied in carotenoprotein extraction to evaluate its operational stability. The immobilized fish trypsin displayed high stability and high catalytic capacity even after 11 re-uses.

Table 5.1: Purification of trypsin from cunner pancreas

	Total Volume (ml)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg)	Yield (%)	Purification Fold
S-1	98.7	746.81	65.33	0.087	100.00	1.00
S-2	97.2	711.74	64.06	0.090	98.06	1.03
Ammonium sulfate fraction (40%-60%)	11.3	81.52	38.87	0.477	59.50	5.45
Acetone fraction	10.4	70.23	47.24	0.673	72.31	7.09
Affinity fraction	16.2	4.80	10.82	2.252	16.55	25.74

Note: Approximately 20 g of pancreatic tissue power was used for the extraction. Data are representative of several other trials.

Table 5.2: Amount of protein loading and enzyme activity of immobilized fish trypsin at various pHs

pH	Protein added (mg/g CPG bead) A	Protein bound (mg/g CPG bead) B	Percentage of protein bound (B/A × 100 (%))	Activity of immobilized enzyme (BAPNA U/g CPG bead)	Specific activity (X 10^{-3} BAPNA U/mg bound protein)
4	40	4.2	10.5	0.117	27.87
5	40	3.8	9.5	0.156	40.97
6	40	20.3	50.8	0.207	10.19
7	40	9.7	24.3	0.174	17.92
8	40	16.4	41.0	0.175	10.67
9	40	27.8	59.5	0.276	9.93
10	40	15.2	38.0	0.194	12.78

Note: Trypsin activities were measured at 25° C with 1 mM BAPNA (in 0.05 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$) as substrate. Specific activity was calculated by dividing activity of immobilized enzyme by the amount of bound protein.

Table 5.3: Kinetic properties of immobilized fish and bovine trypsin

Parameters	Immobilized fish trypsin	Immobilized bovine trypsin
BAPNA		
K_m' (mM)	1.168	1.001
V_{max} (BAPNA Unit / min)	0.398	0.192
V_{max} / K_m'	0.341	0.192
TAME		
K_m' (mM)	1.580	3.688
V_{max} (TAME Unit / min)	3.903	7.502
V_{max} / K_m'	2.470	2.034

Note: Trypsins were assayed for esterase or amidase activity at 25°C as described in the text.

Data are average values for duplicate determinations.

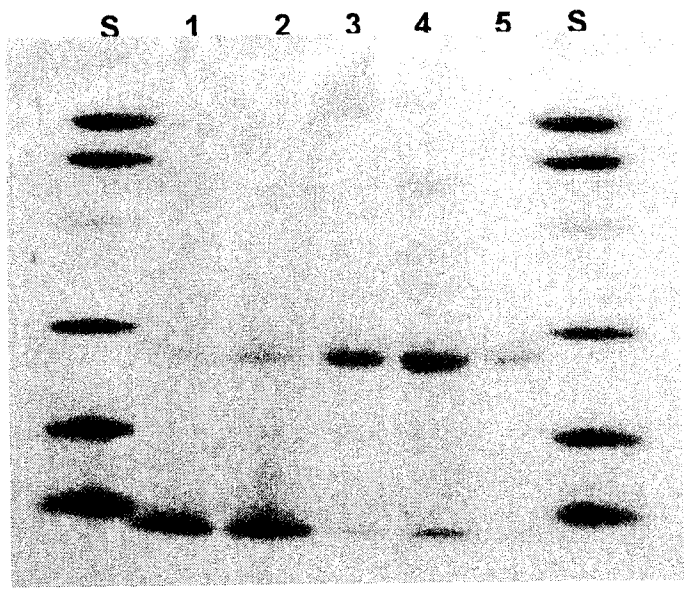


Fig. 5.1: SDS-polyacrylamide gel electrophoresis analysis of cunner fish trypsin.

Lanes: S: protein standards (97.4, 86.2, 45, 31, 21.5, 14.4 kDa); 1: Supernatant-1; 2: Supernatant-2; 3: Ammonium sulfate fraction (40% - 60%); 4: Acetone fraction; 5: Affinity fraction.

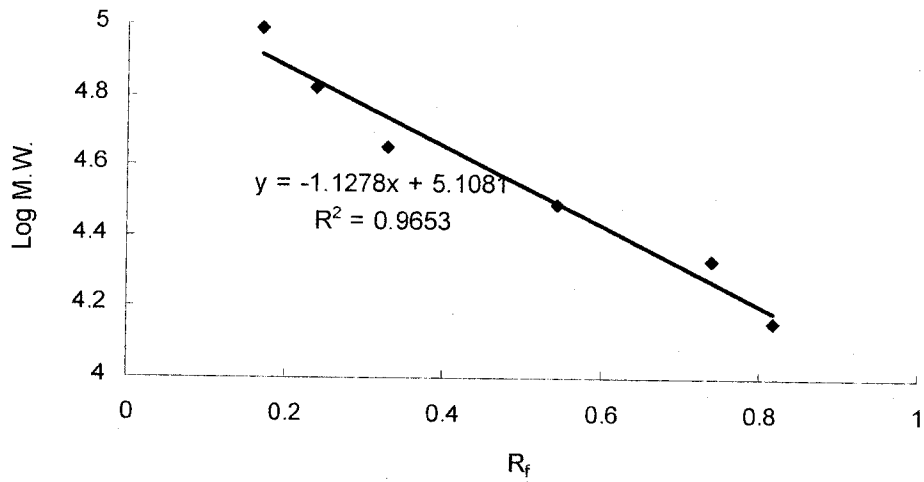


Fig. 5.2: Curve generated by plotting the log of the molecular weight of protein standards vs. R_f.

Note: R^2 is the measure of the fit of the regression line.

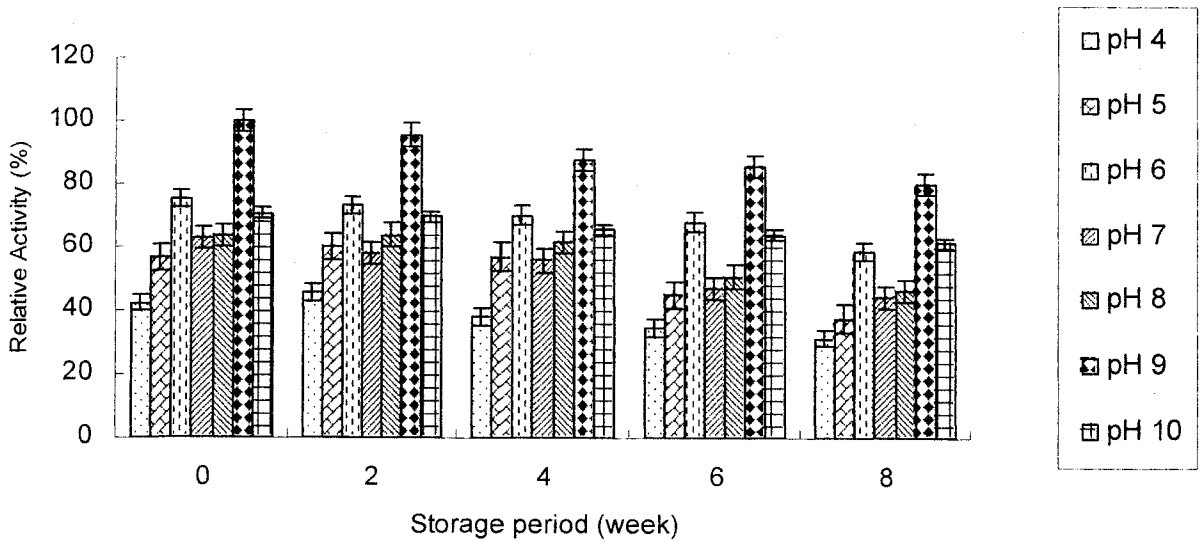


Fig. 5.3: Storage stability of immobilized fish trypsin at different pHs.

Note: 1. Immobilized fish trypsins were stored at 4° C and 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

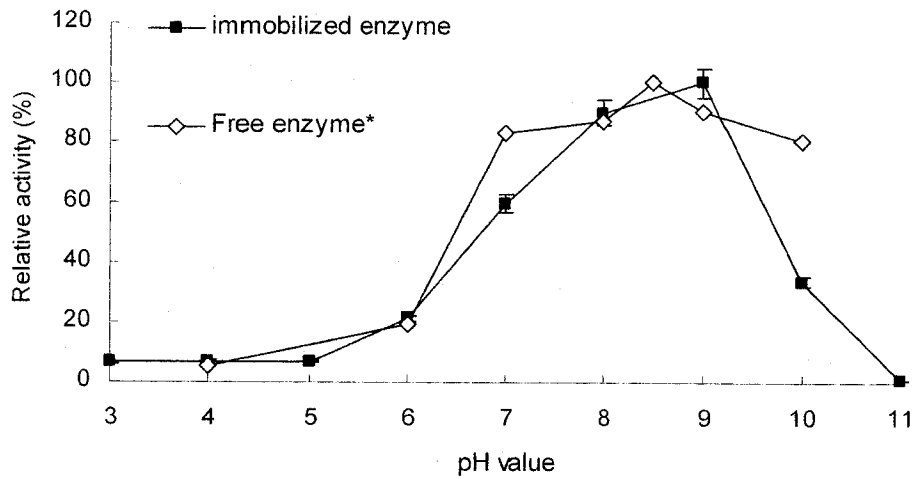


Fig. 5.4: Optimum pH of immobilized fish trypsin on BAPNA at 25°C.

Note: 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate, and reactions were carried out at 25° C.

* Data were adapted from Simpson & Haard (1987).

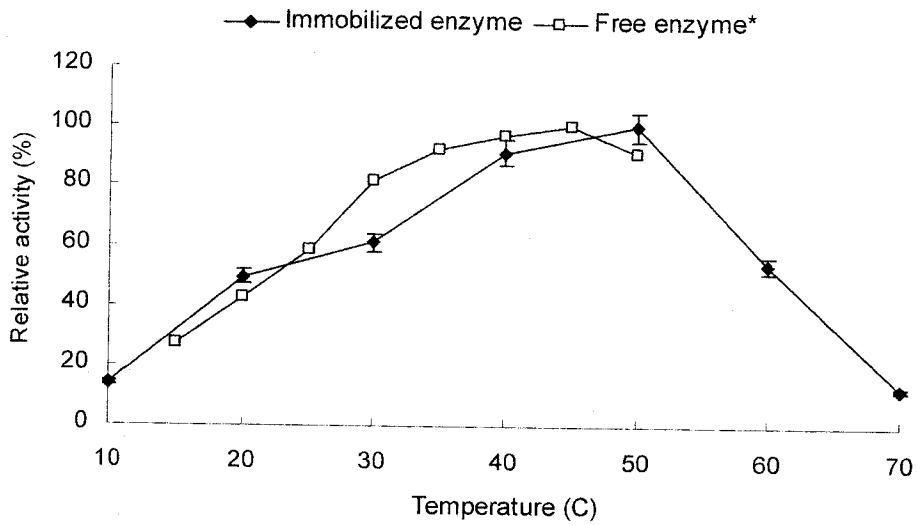


Fig. 5.5: Optimum temperature of immobilized fish trypsin on BAPNA.

Note: 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate, and reactions were carried out as described in the text.

* Data were adapted from Simpson & Haard (1987).

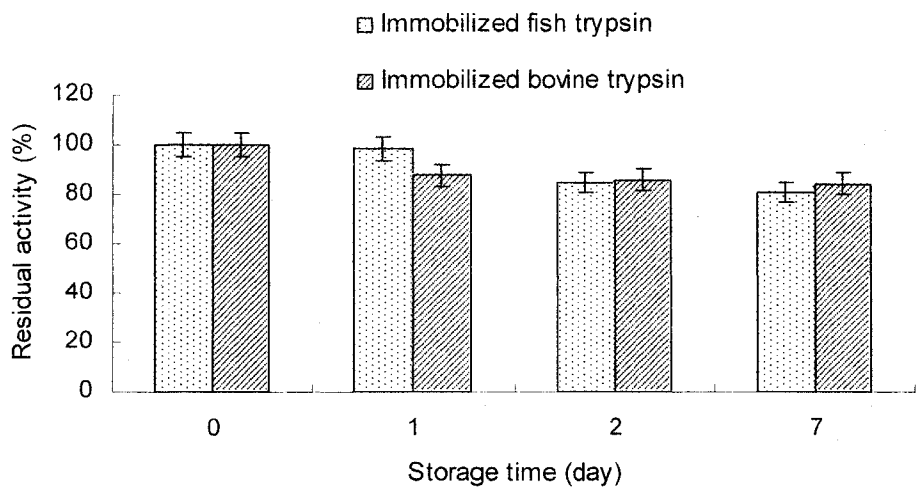


Fig. 5.6: Thermostability of immobilized fish trypsin at 30°C.

Note: 1. 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate, and trypsin activity was assayed at 25° C and pH 8.2.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

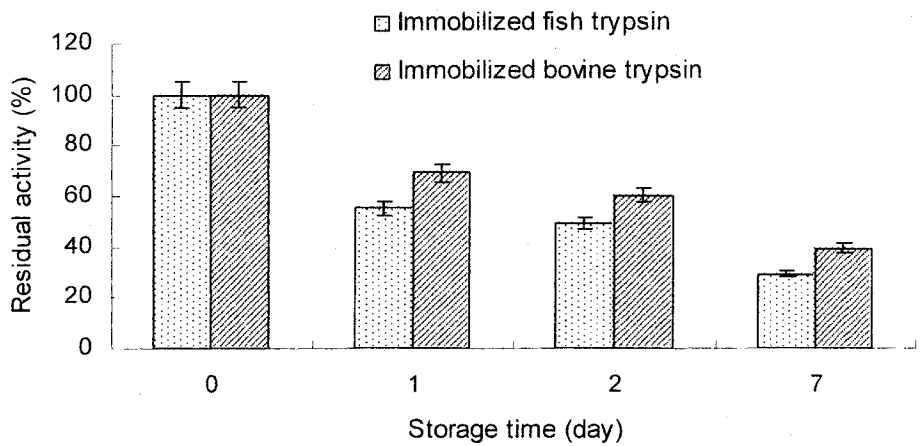


Fig. 5.7: Thermostability of immobilized fish trypsin at 40°C.

Note: 1. 1 mM BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate, and trypsin activity was assayed at 25° C and pH 8.2.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

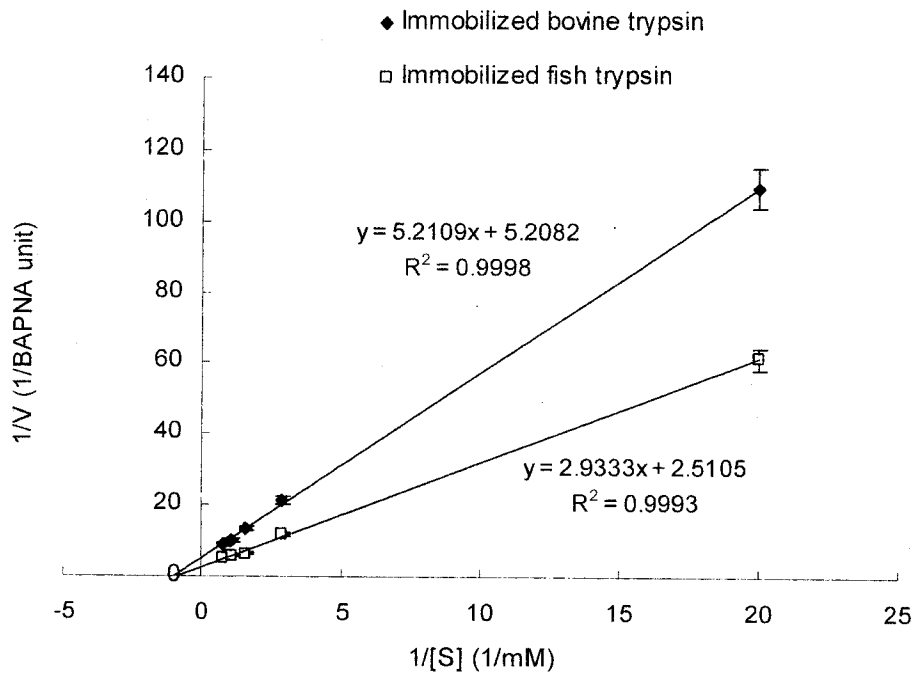


Fig. 5.8: Lineweaver – Burk plots of immobilized fish and bovine trypsin on BAPNA.

Note: 1. BAPNA (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate, and concentrations were in the range of 0.05 mM – 1.25 mM. Trypsin activity was assayed at 25° C and pH 8.2. R^2 is the measure of the fit of the regression line of Lineweaver-Burk equation.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

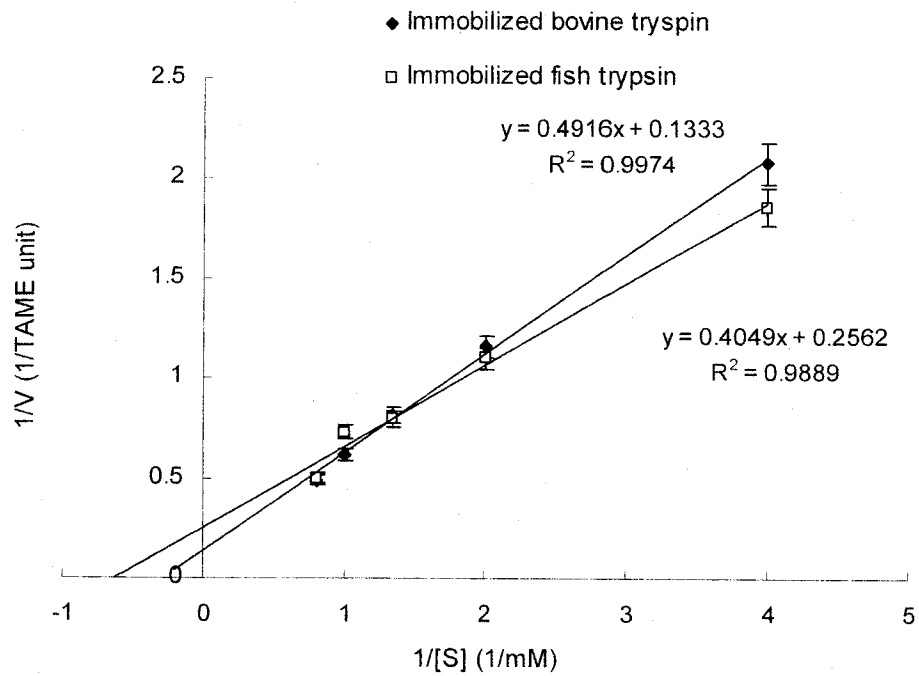


Fig. 5.9: Lineweaver – Burk plots of immobilized fish and bovine trypsin on TAME.

Note: 1. TAME (in 0.05 M Tris-HCl, pH 8.2, containing 0.02 M $CaCl_2$) was used as substrate, and concentrations were in the range of 0.25 mM – 1.25 mM. Trypsin activity was assayed at 25° C and pH 8.2. R^2 is the measure of the fit of the regression line of Lineweaver-Burk equation.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

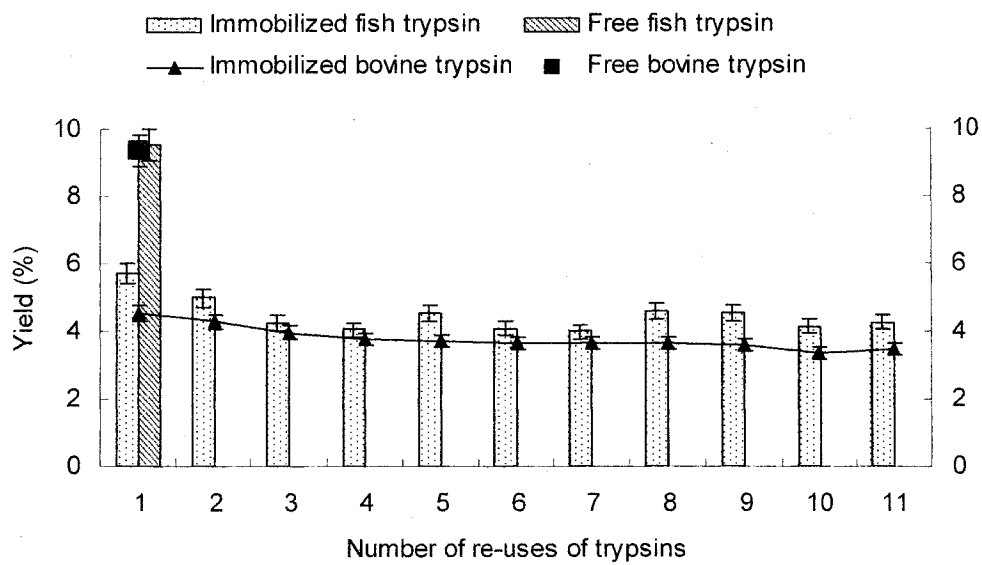


Fig. 5.10: Operational stability of Immobilized fish and bovine trypsin for extracting carotenoprotein from shrimp shell

Note: 1. Yield was based on the dry weight of shrimp shell.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

CHAPTER VI

INACTIVATION OF PECTIN METHYLESTERASE (PME) BY IMMOBILIZED CUNNER FISH TRYPSIN

Connecting Statement

In the previous chapter, the properties of immobilized cunner fish trypsin were studied. The results showed that the immobilized cunner trypsin had high amidase and esterase activities. The following chapter describes an example of application of the immobilized fish trypsin. The immobilized fish trypsin was used to inactivate pectin methylesterase (PME). The inactivation efficiency of immobilized fish trypsin was found to be largely dependent on its enzymatic reaction conditions. The effects of various enzymatic reaction conditions on PME inactivation and the kinetics of PME inactivation using immobilized fish and bovine trypsin were systematically studied in this work.

Note: This chapter constitutes the text of a paper to be submitted for publication as follows:

Li, D. and Simpson, B.K. Inactivation of pectin methylesterase (PME) using immobilized cunner fish trypsin

Contribution of co-author(s): Simpson, B.K. (research / thesis supervisor) gave instructions to the candidate during research, provided financial support for the experiment, and edited the manuscript of this chapter.

Role of candidate: Candidate designed, conducted, analyzed and reported all the experiments presented.

ABSTRACT

Immobilized cunner fish trypsin was used to inactivate pectin methylesterase (PME). The effects of different reaction conditions (*e.g.*, different incubation time, PME concentration, and reaction temperature) on PME inactivation, and the kinetics studies of PME inactivation were investigated. Temperature, incubation time, PME concentration and the interaction between these factors significantly affect the extent of PME inactivation. In general, higher temperature, longer incubation time, and lower initial PME concentration would cause more PME inactivation. Moreover, the immobilized fish trypsin had higher capacity to inactivate PME than immobilized bovine trypsin. The inactivation efficiency of the immobilized fish trypsin was 20% higher than that of the immobilized bovine trypsin. PME inactivated by immobilized fish trypsin and bovine trypsin regained part of its activity during storage at 4°C, with the PME treated by immobilized fish trypsin regaining much more of its original activity than PME treated by immobilized bovine trypsin. Furthermore, heat-denatured PME was inactivated by immobilized fish trypsin more extensively than by immobilized bovine trypsin. The kinetic studies indicated that the inactivation rate constants increased and *D*-values decreased with increasing temperature for both immobilized fish trypsin and bovine trypsin. The inactivation rate constants of immobilized fish trypsin at every temperature (*e.g.*, 15°C, 25°C, 35°C) were higher than those of immobilized bovine trypsin. The activation energy (E_a) of PME inactivation by immobilized fish trypsin was lower than that of immobilized bovine trypsin, which explains why the immobilized fish trypsin had higher catalytic capacity at various

temperatures than immobilized bovine trypsin.

Key words: cunner fish trypsin, immobilization, inactivation, pectin methylesterase (PME), kinetic properties

INTRODUCTION

Pectin methylesterase (PME, EC 3.1.1.11) is widely distributed in fruits and vegetables. It is bound to the cell walls by electrostatic interaction (Ly-Nguyen *et al.*, 2002a) and catalyzes the de-esterification of methyl esters of polygalacturonic acid polymers to form pectinic acids / pectic acids and methanol. In citrus juice, PME hydrolyzes pectin (methyl esters of polygalacturonic acid) and transforms it gradually into low-methoxy pectin or pectin acids. This de-esterification process can cause a lot of problems, such as lowering the viscosity of juice, and the formation of insoluble complex formed by low-methoxy pectin and calcium, which results in the loss of cloudy appearance of juice (Van de Broeck *et al.*, 1999).

To prevent undesirable changes of the juice by PME, heat treatment, such as blanching, pasteurization and commercial sterilization, is commonly applied to inactivate PME, (Anthon & Barrett, 2002). However, PME is relatively thermostable, thus severe heating conditions are necessary to inactivate this enzyme (Van de Broeck *et al.*, 2000a). Unfortunately, such severe heat treatment of juice can cause undesirable quality losses, such as changing color, texture, flavor, and nutrients (*e.g.*, ascorbic acid) (Fachin *et al.,* 2002), consequently, minimum thermal treatment is preferred for preserving high quality of the raw material (Ly-Nguyen *et al.,* 2002b).

Due to the undesirable side effects of PME inactivation by thermal treatment, other nonthermal treatments such as high pressure (Van de broeck *et al.*, 2000a,

2000b; Riahi & Ramaswamy, 2003; Ly-Nguyen *et al.*, 2002c), low pH values (Owusu-Yaw *et al.*, 1998), PME inhibitors (Ly-Nguyen *et al.*, 2004); pulsed electric fields (Anthon & Barrett, 2002), nitrogen bubbling (Caussette *et al.*, 1998) have been proposed. However, every method still has its own drawbacks. For example, low pH method can cause loss of almost all the vitamin C, high pressure method has the problem of enzyme reactivation, and using PME inhibitors has the possibility of enzyme reactivation when the inhibiting effect is removed (Kyei, 1997).

To produce high-quality juice and fruit-based products, an effective approach to inactivate PME, and minimize quality loss of the juice at the same time becomes necessary (Van de Broeck *et al.,* 1999). To overcome the drawbacks of the above methods, researchers have tried to combine two or three methods for more efficient PME inactivation. Pressure-temperature inactivation of PME is the most commonly used method (Ly-Nguyen *et al.,* 2003; Hernandez & Cano, 1998; Crelier *et al.*, 2001). PME inhibitors and temperature or pressure have also been used to inactivate PME (Ly-Nguyen *et al.*, 2004). Nitrogen bubbling and temperature were used in combination to inactivate PME by Caussette *et al.,* (1998). Although some promising results have been obtained, studies are not fully satisfied with the current methods for PME inactivation.

Another possible alternative is using digestive proteases, especially, the digestive proteolytic enzymes from cold water fish or stomachless fish to inactivate PME (Kyei, 1997; Simpson, 2000). Because this kind of protease exhibits higher

stability at low temperature and possesses higher catalytic ability on native proteins compared with similar enzymes from mammals, thermophilic organisms and plant sources (Simpson & Haard, 1985, 1987). By using the digestive protease from stomachless fish, PME can be hydrolyzed and inactivated under mild conditions without any serious quality loss caused by those thermal or other physical treatments (Simpson, 2000).

With the enzyme immobilization technique, the protease can be reused and the enzyme reaction can be carried out in a continuous way (Lee & Swaisgood, 1997; Brena *et al.*, 2003). The most important benefit of enzyme immobilization is non-contamination in the final products, and thereby reducing the overall manufacturing cost by eliminating the need to separate the enzyme from the final products (Bickerstaff, 1997a; Brena, *et al.*, 2003).

MATERIAL AND METHODS

Materials

Controlled pore glass beads (CPG2000A, surface area 11 m^2/g, bead mesh range 80/120) were purchased from CPG Inc, Lincoln Park, NJ. *N*-α-benzoyl-*DL*-arginine-*p*-nitroanilide (BAPNA), *p*-toluene-sulfonyl-*L*-arginine methyl ester (TAME), dimethyl sulfoxide (DMSO), 3-aminopropyltriethoxysilane, glutaraldehyde, bovine trypsin (type III), soybean trypsin inhibitor, nitric acid, ammonium sulfate, cyanogen bromide (CNBr) activated Sepharose-4B, polyoxyethylene lauryl ether (Brij 35), pectin methylesterase (PME), pectin (from

citrus fruits) were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Sodium borate, sodium acetate, sodium chloride, hydrochloric acid, monobasic and dibasic sodium phosphate, tris (hydroxymethyl) aminomethane (Tris), tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), acetone, potassium phosphate, potassium phosphate dibasic, and sodium hydroxide were purchased from Fisher Chemicals (Nepean, Ontario, Canada). Bromothymol blue was purchased from ACP Chemicals Inc. (Montreal, Quebec, Canada), and galacturonic acid was purchased from ICN Biomedicals Inc. (Toronto, Ontario, Canada)

Biological specimens

Live cunner fish were caught by a fisherman at the coast of Miscou (New Brunswick, Canada.) and transported live to the lab where they were held in tanks till needed.

Extraction and purification of trypsin from cunner

Trypsin fraction from cunner fish pancreas was prepared according to the method of Simpson and Haard (1985). The pancreatic tissue was taken from fish and was rapidly frozen in liquid nitrogen, and powdered in a waring blender. About 10 g of the tissue powder were mixed with 0.05 M Tris-HCl buffer (pH 7.8) containing 0.02 M $CaCl_2 \cdot 2H_2O$, at 4°C for 3 h (the ratio of tissue and buffer was 1:5 w/v). The homogenate was centrifuged at 3,000 g in a J2-21 centrifuge (Beckman Coultier Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C to

remove insoluble material and obtain the first supernatant (Sup 1). Sup 1 was collected and was made up to 0.2% with Brij 35, and stirred at 4°C for 3 h, and then centrifuged at 10,000 g (in a J2-21 centrifuge, Beckman Coultier Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C to obtain the second supernatant (Sup 2). Sup 2 was fractionated with solid ammonium sulfate and the fraction precipitating between 40% and 60% saturation was collected by centrifugation at 6,000 g (in a J2-21 centrifuge, Beckman Coultier Canada Inc, Ville Saint-Laurent, QC, Canada) for 30 min at 4°C. The $(NH_4)_2SO_4$ precipitate was dissolved in 20 ml of 0.05 M Tris-HCl buffer (pH 7.8, containing 0.02 M $CaCl_2$) and dialyzed for 12 h against three changes of 6 L of the Tris-HCl buffer to constitute the ammonium sulfate fraction. The dialysate was mixed with three times its volume of cold acetone (-20°C) and kept at -20°C for 3 h. This precipitate formed was collected by centrifugation at 6,000g (in a J2-21 centrifuge, Beckman Coultier Canada Inc, Ville Saint-Laurent, QC, Canada) at 4°C for 30 min, and then redissolved in 10 ml of the Tris-HCl to form the acetone fraction. The acetone fraction (10 ml) was pumped (Micro tube pump MP-3, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at a rate of 15 ml / h onto a soybean trypsin inhibitor (SBTI)-Sepharose 4B affinity chromatography column (that had been prepared as the procedure of Pharmacia Fine Chemicals; Anonymous, 1983). The column was thoroughly washed with elution buffer (0.05 M Tris-HCl buffer, pH 7.8, containing 0.02 M $CaCl_2$) to remove the unbound material after which the bound material was eluted with 5 mM HCl at a rate of 15 ml / h, and fractions of 4.8 ml / tube were collected in test. The fractions showing amidase activity were pooled together and adjusted to pH 7.8 with Tris-HCl buffer (as the affinity

fraction) and stored frozen at -20°C.

Immobilization of cunner fish trypsin

The immobilized cunner fish trypsin was prepared based on the method of Janolino & Swaisgood (1997) and Sear & Clark (1993). Controlled pore glass (CPG) beads were washed by concentrated nitric acid. Preciously used glass beads were pre-heated at 600°C for 24 h before washing with nitric acid. Then the beads were derivatized as aminopropyl-CPG with 10% aqueous solution of 3-aminopropyltriethoxysilane (1:3 w/v CPG to 3-aminopropyltriethoxysilane solution). Further, 10% glutaraldehyde was used to modify the beads to glutaraldehyde-CPG (1:10 w/v CPG to glutaraldehyde solution). The activated glass beads were then added to the enzyme solution, which was incubated with glass beads for 21 h at 4°C. Next, the glass beads were washed with 100 mM sodium borate / 1 M sodium chloride, pH 8.5, and 100 mM sodium acetate / 1 M sodium chloride, pH 4.75 (1:250 w/v CPG to buffer). The washings were repeated for 4 times as above and finally the CPG beads were washed with 10 mM sodium phosphate, pH 7.3 (1:1000 w/v CPG to buffer). The damp beads were collected and lyophilized to dryness (in a LYPH-LOCK 12 freeze dry / shell freeze system, Labconco, Kansas City, Missouri).

Determination of pectin methylesterase (PME) activity

PME activity was determined by the method of Hagerman and Austin (1986). The pH values of all the solutions – pectin, indicator dye (bromothymol blue), and

distilled water were adjusted to pH 7.5 using 2 M NaOH before each assay. A 0.5% (w/v) solution of pectin was prepared in distilled water by heating the mixture at 40°C with continuous stirring. The pectin solution was stored at 4°C. A 0.01% (w/v) bromothymol blue solution was prepared in 0.003 M potassium phosphate buffer, pH 7.5. The reaction was monitored at 620 nm with a spectrophotometer (Hitachi U 2000, Tokyo, Japan). About 2.00 ml of the pectin solution was mixed with 0.60 ml of bromothymol blue solution and 0.83 ml of distilled water. Distilled water was used as blank to zero the spectrophotometer. The initial absorbance at 620 nm (A_{620}) of the mixture, approximately 0.28, remained constant until 20 µl of PME solution was added. The rate of decrease in absorbance at 620 nm was recorded. The method was calibrated with appropriate dilutions of a 0.87 mM galacturonic acid.

Inactivation of PME with immobilized fish / bovine trypsin

The effects of reaction temperature, PME solution concentration, and the incubation time for the treatment of the PME solution with the immobilized fish / bovine trypsin were studied. The applied reaction temperatures were 15°C, 25°C, 35°C; the PME concentration used were 0.25 mg/ml, 0.75 mg/ml, 1.25 mg/ml, and the incubation times of the PME with the enzymes were 1.5 h, 2.5 h and 3.5 h, respectively (Table 6.1). About 1 g of the immobilized fish / bovine trypsin was added to various concentrations of the PME solution. The mixtures were shaken in a water-bath shaker (130 rpm, Shaking Water Bath 25, Precision Scientific, Chicago, IL, US) at the selected temperatures. After 1.5 h, 2.5 h and

3.5 h intervals, 50 µl of samples were taken from the mixtures, and the residual activities of PME solutions were measured by the method of Hagerman and Austin (1986).

Reactivation studies of immobilized fish / bovine trypsin treated PME

PME solutions with concentrations of 0.25 mg/ml, 0.75 mg/ml, and 1.25 mg/ml were treated by immobilized fish / bovine trypsin at temperatures of 15°C, 25°C and 35°C for 4 h. The trypsin treated PME samples were stored at 4°C for 18 days, and PME residual activity was tested every 3 days by the method of Hagerman and Austin (1986).

Inactivation of heat-treated PME with immobilized fish / bovine trypsin

PME solutions with concentration of 0.6 mg/ml were incubated at 40°C and 50°C for 30 min, and then immediately cooled and stored on ice at least for 15 min. Equal amounts of immobilized fish / bovine trypsins were added to the heat-treated PME, and the mixtures were incubated at 25°C for 4 h. About 50 µl of PME samples were taken at 1 h intervals and the residual PME activity was assayed with the method of Hagerman and Austin (1986).

Kinetic studies of PME inactivation by immobilized fish / bovine trypsin

The experiments were performed in a water-bath shaker (130 rpm, Shaking Water Bath 25, Precision Scientific, Chicago, IL, US). The temperatures were set

at 15°C, 25°C, and 35°C, respectively. About 1 g of immobilized fish / bovine trypsin was added to 5.0 ml of PME solution (0.25 mg/ml). After 60 min incubation, 50 µl of PME samples were taken from the mixture after 30 min intervals, and immediately cooled on ice. The residual PME activity was assayed with the method of Hagerman and Austin (1986).

Data analysis

Inactivation of enzymes can be described by a first-order kinetics model, which shows that enzyme activity decreased logarithm-linearly with time (Van de Broeck *et al.,* 2000a):

$$\ln(A_t / A_0) = -kt \qquad (6-1)$$

where A_t is the enzyme activity at time t, A_0 is initial enzyme activity, t is treatment time, and k is the first-order inactivation rate constant. This relation is valid under isothermal and isothermal – isobaric conditions.

A special case of the first-order model is a fractional conversion model. According to Ly-Nguyen *et al.,* (2002b), the fractional conversion f takes into account a nonzero activity after prolonged treatment ($=A_\infty$) and can be expressed mathematically as:

$$f = \frac{(A_0 - A_t)}{(A_0 - A_\infty)} \qquad (6-2)$$

To account for the nonzero activity after prolonged treatment, fractional conversion in the following form should be used:

$$\ln(1-f) = \ln(\frac{A_t - A_\infty}{A_0 - A_\infty}) = -kt \qquad (6-3)$$

Equation (6 – 3) may be re-arranged further as shown in Eq. (6 – 4). By plotting A_t (activity after different time intervals) versus inactivation time at constant pressure and / or temperature conditions, the inactivation rate constant, k, and remaining activity, A_∞ can be estimated using nonlinear regression analysis:

$$A_t = A_\infty + (A_0 - A_\infty)\exp(-kt) \qquad (6-4)$$

For most irreversible fist-order reactions, A_∞ approaches 0 and Eq. (6 – 2) can be reduced to

$$f = \frac{(A_0 - A_t)}{A_0} \qquad (6-5)$$

A plot of the logarithm of (1 – f) versus time yields a straight line with a rate constant expressed by the negative slope value:

$$\ln(A_t / A_0) = \ln(1-f) = -kt \qquad (6-6)$$

So, Eq. (6 – 6) is identical to Eq. (6 – 1), when A_∞ approaches 0.

Once inactivation rate constants, k, at different temperatures are known, the activation energy (E_a) of enzyme inactivation can be estimated using the Arrhenius relationship:

$$\ln k = k_{ref} + [\frac{E_a}{R}(\frac{1}{T_{ref}} - \frac{1}{T})] \qquad (6-7)$$

A reference temperature was chosen near the middle of the temperature range used for inactivation determinations. The rate constant of inactivation at this reference temperature (k_{ref}) was determined from the value of ln(k_{ref}) at this temperature given by the regression line in the Arrhenius plot (Anthon & Barrett, 2002).

The D value, the time required to reduce the enzyme to 10% of its original value also can be calculated from the inactivation rate constant *k* by Eq. (6 – 8) (Fachin *et al.*, 2002).

$$D = 2.303 / k \qquad (6-8)$$

RESULTS AND DISCUSSION

Inactivation of native PME by immobilized fish / bovine trypsin

The studies on PME inactivation by immobilized fish / bovine trypsin were carried out at different temperatures, incubation time, and PME initial concentrations.

The inactivation of PME by both immobilized fish trypsin and immobilized bovine trypsin was affected by the incubation temperature, incubation time, and the initial concentration of PME as shown in Fig. 6.1 and Fig. 6.2.

In general, higher temperature, longer incubation time, and lower initial PME concentration caused more PME inactivation. However, it was observed that at some points, the results showed an opposite trend. For instance, when PME concentration was 1.25 mg/ml, PME inactivation efficiency of immobilized fish trypsin at 15° C was higher than that at 25° C (Fig. 6.1), which indicated that the immobilized fish trypsin showed high PME inactivation efficiency at low temperature (*e.g.*, 15° C), especially the PME with high concentration (*e.g.*, 1.25 mg/ml)

ANOVA of the data indicated that the factors of incubation time, temperature and PME concentration were highly significant ($p < 0.001$) at the 99% confidence level for both immobilized fish and bovine trypsin (Table A2-1). All the interactions between these factors were also highly significant ($p < 0.001$) at the 99% confidence level for both of immobilized fish and bovine trypsin (Table A2-1). Therefore, to control PME inactivation by fish / bovine trypsin, all the above factors and the interactions between these factors should be considered.

Furthermore, the inactivation capacities of the immobilized fish and bovine trypsins are statistically significant at the 99% confidence level ($p < 0.001$), and the immobilized fish trypsin had higher capacity for PME inactivation compared with immobilized bovine trypsin. On the average, the PME inactivation capacity of the immobilized fish trypsin was 10 times higher than that of the immobilized bovine trypsin, especially at relatively lower temperatures (*e.g.*, 15°C). This observation supports the findings previously made by Simpson and Haard (1985)

stating that the protease from this particular stomachless fish had more catalytic capacity on native protein substrate.

Reactivation of PME inactivated by immobilized fish / bovine trypsin

PME solutions with different initial concentrations were treated with immobilized fish / bovine trypsins at different temperatures and incubated for 4 h. The immobilized trypsin treated PME was then stored at 4°C for 18 days to study the reactivation of the inactivated PME. The results were shown in Fig. 6.3. Overall, the PME treated by immobilized bovine and fish trypsin could regain activity during storage at 4°C.

Although immobilized fish trypsin inactivated PME to a greater extent than immobilized bovine trypsin did, PME treated by immobilized fish trypsin exhibited slightly higher extent of reactivation than the PME treated by immobilized bovine trypsin. For example, at PME concentration of 0.25 mg/ml, PME treated by immobilized fish trypsin at 15° C regained 20% of its original activity after 3 day storage at 4° C. The same PME solution treated by immobilized bovine trypsin recovered 15% of its original activity after 3 day storage at 4°C. Moreover, PME inactivated at higher temperature (*e.g.*, 35°C) showed less ability of reactivation than PME inactivated at lower temperatures (*e.g.*, 15°C and 25°C). For instance, PME inactivated by immobilized fish trypsin at 15° C regained its 11% of its original activity after 6 days of storage, however, the same PME inactivated at 25°C and 35°C showed 9% and 7% reactivation, respectively.

The initial concentration of PME also affected the reactivation of PME. PME with concentration of 0.25 mg/ml treated by immobilized trypsin showed its strong reactivation during 3 days of storage. Higher concentration of PME prolonged the reactivation period. When the PME concentration was 0.75 mg/ml and 1.25 mg/ml, the treated PME exhibited strong reactivation during 6 days of storage.

Even though it is obvious that PME treated by immobilized fish trypsin regained some activity during storage, PME inactivation by immobilized fish trypsin is still worth pursuing, because it is effective under mild conditions. For example, 70% of PME activity can be lost after 3.5 h of treatment by immobilized fish trypsin at 25°C. Compared with thermal PME inactivation, and thermal and high-pressure combined treatment, 50% of PME activity was lost at 55°C after 10 min treatment (Ly-Nguyen *et al.*, 2002c), and 65% of PME activity was lost using a combined treatment of 150 MPa/30°C during 15 min (Hernandez & Cano, 1998), the inactivaton achieved in this study is comparable or better than the other methods. In addition to the mild conditions, enzyme immobilization also makes the enzyme reusable and minimizes the contamination of enzyme in the final products, thus increases the quality of the final product and reduces the overall processing cost (Boadi & Neufeld, 2001; Zhu *et al.*, 2005a).

Inactivation of partially heat-denatured PME by immobilized fish / bovine trypsin

To investigate the inactivation of partially heat-denatured PME, PME solution was treated by immobilized fish / bovine trypsin at various temperatures, such as 40°C, 50°C, and 60°C. After 30 min incubation at 40°C, the residual activity of PME was 57.66%, and after 30 min incubation at 50°C, the residual activity of PME was 47.62%. However, after 30 min incubation at 60°C, the residual activity of PME was 8.17% (Fig. 6.4 a). PME solutions treated at 40°C, 50°C for 30 min were chosen as samples, subsequently reacted with immobilized fish / bovine trypsin for further inactivation.

Fig. 6.4 shows the PME inactivation increased with incubation time and inactivation by the immobilized fish trypsin was more extensive than with immobilized bovine trypsin. For the PME sample (heat-denatured at 40°C for 30 min) treated with immobilized fish trypsin for 4 h, its residual activity reduced from 57.66% to 25.47%. However, the residual activity of the same PME sample after 4 h incubation with immobilized bovine trypsin only decreased from 57.66% to 38.14%.

It is obvious that the longer incubation period, the more PME inactivation. For instance, the PME sample (heat-denatured at 40°C for 30 min) treated with immobilized fish trypsin for 1 h, 2 h, 3 h, and 4 h, the activity dropped from 57.66% to 46.41%, 38.61%, 32.31% and 25.46%, respectively. Similarly, the

PME sample (heat-denatured at 50°C for 30 min) treated with immobilized fish trypsin for 1 h, 2 h, 3 h, and 4 h, the activity decreased from 47.62% to 34.68%, 27.28%, 20.53% and 10.67%, respectively. It is mentioned here also that the PME sample (heat-denatured at 40°C for 30 min) treated with immobilized fish trypsin for 4 h had a final activity of 25.46% compared with the sample (heat-denatured at 50°C for 30 min) treated for 4 h that had a final activity of 10.67%. Thus, the higher temperature (50°C) achieved greater inactivation than the lower temperature. The possible reason for this phenomenon is the effect of denaturation extent of PME on PME inactivation by immobilized fish trypsin. The condition of 50°C, 30 min made protein / enzyme more denatured compared with the condition of 40°C, 30 min.

Kinetic studies of the inactivation of native PME by immobilized fish / bovine trypsin

PME inactivation by immobilized cunner fish / bovine trypsins was carried out at 15°C, 25°C and 35°C. The inactivation process of both immobilized fish trypsin and immobilized bovine trypsin was found to be the first order reaction with respect to active PME (Figs. 6.5 & 6.6). Inactivation rate constants (k) and D-values, estimated from the linear regression of $\ln(A_t / A_o)$ versus t, are shown in Table 6.2. As expected, the inactivation rate constants increased and D-values (time to inactivate 90% of the enzyme) decreased with increasing temperatures for both immobilized fish trypsin and immobilized bovine trypsin. Moreover, the inactivation rate constants of immobilized fish trypsin at various temperatures

(*e.g.*, 15°C, 25°C and 35°C) were higher than those of immobilized bovine trypsin, which indicated that the immobilized fish trypsin had higher catalytic capacity on PME at the different temperatures. Simpson and Haard (1987) reported that the free form of cold water fish trypsin had high activity at relatively low temperature. In this research, the immobilized form of fish trypsin also showed higher catalytic capacity on PME at relatively low temperature (*e.g.*, 15°C). Ly-Nguyen and his co-workers (2002c) reported that the inactivation rate constants of purified carrot PME at 10°C by high pressure (600 MPa, 625 MPa) were 0.0034 min^{-1} and 0.0045 min^{-1}, which are close to the inactivation rate constants obtained from immobilized fish trypsin (*e.g.*, 0.0042 min^{-1} for 15°C, 0.0052 min^{-1} for 25°C and 0.0056 min^{-1} for 35°C).

For both immobilized fish trypsin and immobilized bovine trypsin, the inactivation rate constant increased with increasing temperature (Table 6.2). However, it was noticed that at 35°C, the inactivation rate constant of immobilized fish trypsin is just slightly higher than that at 25°C. In contrast, the inactivation rate constant of immobilized bovine trypsin at 35°C is much higher than that at 25°C. This phenomenon could be explained as follows: high temperatures speed up the chemical reactions in most cases. The reaction rate increases with increasing temperature. However, previous studies showed that the immobilized fish trypsin showed relatively lower thermostability compared with immobilized bovine trypsin. Thus, immobilized fish trypsin may lose part of its catalytic capacity at high temperatures (*e.g.*, 35°C) and long incubation period (*e.g.*, 4 h), so that the inactivation rate constant values of immobilized fish trypsin at 35°C did not

increase as much as expected. The *D*-values for both immobilized fish trypsin and immobilized bovine trypsin showed a similar trend to that of the inactivation rate constant.

From the above discussion, it is inferred that the immobilized fish trypsin was more heat-labile compared with the immobilized bovine trypsin, especially for long incubation period.

From the Arrhenius plot of the rate constant versus *1/T* (Fig. 6.7), the activation energies (E_a) of PME inactivation by immobilized fish trypsin and immobilized bovine trypsin were obtained (Table 6.2). The activation energy of the PME inactivation by immobilized fish trypsin (E_a = 2970.2±693.8 kJ/mol) was lower than that of immobilized bovine trypsin (E_a = 26068.4±2226.5 kJ/mol). The activation energy for immobilized bovine trypsin is 9 times higher than the immobilized fish trypsin, which could be the reason of the high catalytic capacity of immobilized fish trypsin at lower temperatures. The differences between the activation energy of the immobilized fish and bovine trypsin could be explained by the subtle structural differences of fish trypsin molecules and bovine trypsin molecules (Ciardiello, *et al.*, 2000; Clare, *et al.*, 2001).

Genicot *et al.*, (1996) compared the structural features of the binding pockets of the bovine and fish (Antarctic fish) trypsins, which revealed that fish trypsin lacks Tyr-151, and in bovine trypsin, residue Gln-192 is located at the entrance of the binding pocket and there was only 3.2 Å in distance. In addition, the residue next

to Gln-192 is a proline in bovine trypsin, which is substituted by another residue glycine with smaller size in fish trypsin (Genicot *et al.*, 1996). Macouzet (2004) revealed the amino acid sequence of cunner fish trypsin, and the identities of nucleotides and amino acid with Antarctic fish was 90% and 86%, respectively, and the main residues mentioned above were included. Therefore, the above theory could also be applied to cunner fish trypsin with high catalytic efficiency.

In fish enzyme, the residue Gln-192 has a lot of freedom comparing to that in bovine trypsin, which makes the binding pocket of fish trypsin much more flexible. As a result, the fish trypsin is able to adjust the structure of its binding pocket by rotating its flexible Gln-192 residue and thus has the ability to catalyze the reactions of substrate with various shapes and sizes. In contrast, the rigid binding pocket of bovine trypsin makes it only suitable to catalyze the reactions of substrate with a specific shape and size. In consequence, the fish trypsin had higher catalytic capacity than bovine trypsin at relatively low temperatures (*e.g.*, 15°C) and mild temperatures (*e.g.*, 25°C and 35°C), and its enzymatic rate constants were higher.

CONCLUSIONS

Inactivation of PME using immobilized fish trypsin was effective, and was influenced by the PME concentration, incubation time and temperature. Overall, lower PME concentration, longer incubation period, and higher temperature enhanced PME inactivation. The immobilized fish trypsin inactivated PME more

extensively than immobilized bovine trypsin did. The PME inactivated by immobilized fish and bovine trypsin regained part its activity during storage at 4°C. The concentration of PME solution affected the reactivation period. The reactivation period increased from 3 days to 6 days when PME concentration increased from 0.25 mg /ml to 0.75 mg /ml or 1.25 mg/ml. Partially heat-denatured PME inactivation strongly depends on the extent of PME denaturation and the incubation time. Higher denaturation extent and longer incubation time lead to higher extent of PME inactivation. The inactivation of partially heat-denatured PME by immobilized fish trypsin was more extensive than that by immobilized bovine trypsin. The kinetic studies showed that the inactivation rate constants increased and *D*-values decreased with increasing temperature for both immobilized fish and bovine trypsins. The inactivation rate constants of immobilized fish trypsin at various temperatures were higher than those of immobilized bovine trypsin. From the Arrhenius plot, the activation energy of the PME inactivation process by immobilized fish trypsin was smaller than that by immobilized bovine trypsin, which could explain the high catalytic capacity of the immobilized fish trypsin at various temperatures.

Table 6.1: Experimental plan* for PME inactivation by immobilized fish / bovine trypsin

Assay	PME concentration (mg/ml)	Incubation time (h)	Temperature (°C)
1	0.25	1.5	15
2	0.25	2.5	15
3	0.25	3.5	15
4	0.75	1.5	15
5	0.75	2.5	15
6	0.75	3.5	15
7	1.25	1.5	15
8	1.25	2.5	15
9	1.25	3.5	15
10	0.25	1.5	25
11	0.25	2.5	25
12	0.25	3.5	25
13	0.75	1.5	25
14	0.75	2.5	25
15	0.75	3.5	25
16	1.25	1.5	25
17	1.25	2.5	25
18	1.25	3.5	25
19	0.25	1.5	35
20	0.25	2.5	35
21	0.25	3.5	35
22	0.75	1.5	35
23	0.75	2.5	35
24	0.75	3.5	35
25	1.25	1.5	35
26	1.25	2.5	35
27	1.25	3.5	35

* Factorial design was used for this experiment.

Table 6.2: Kinetic parameter estimates of a first-order inactivation of PME by immobilized cunner fish trypsin and immobilized bovine trypsin.

Temperature (°C)	k (min^{-1})	R^2	D (min)
Immobilized cunner fish trypsin			
15	0.0042±0.00006[a]	0.92	552.8±7.7
25	0.0052±0.0002	0.96	434.8±13.9
35	0.0056±0.0003	0.99	414.5±22.4
E_a=2970.2±693.8 kJ/mol		R_{Ea}^2 = 0.91[b]	
Immobilized bovine trypsin			
15	0.0002±0.00006	0.97	10235.6±2216.1
25	0.0009±0.00006	0.92	2473.6±147.7
35	0.0029±0.00006	0.99	785.3±15.3
E_a=26068.4±2226.5 kJ/mol		R_{Ea}^2 = 0.99[b]	

[a]Standard error of regression.
[b]Regression coefficient of the Arrhenius equation.

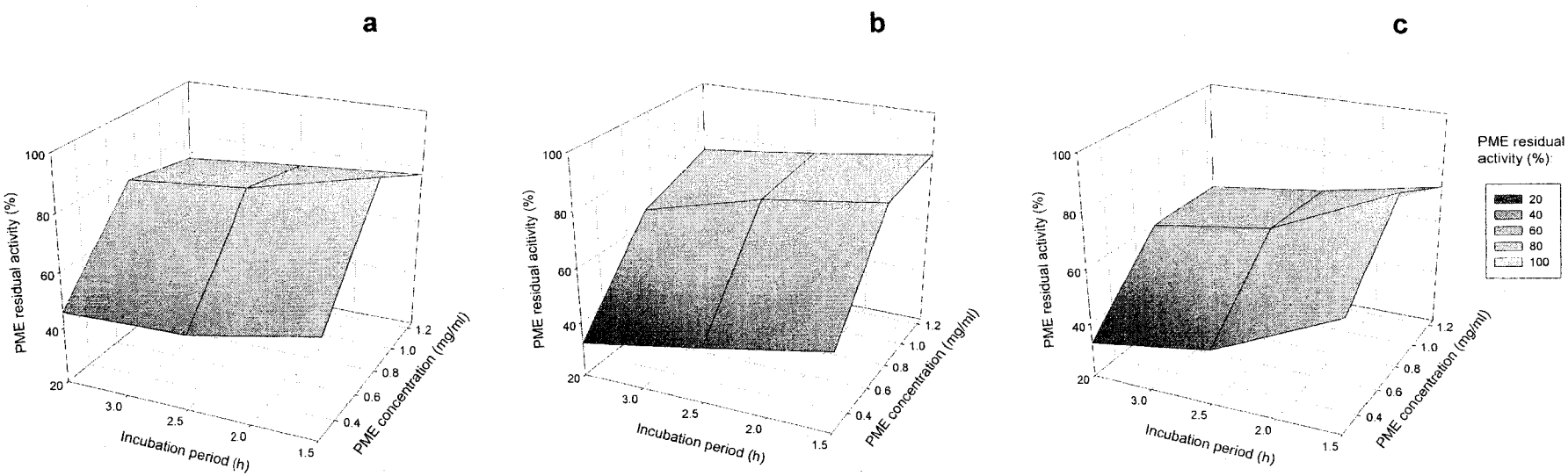


Fig. 6.1: Residual activity of PME treated by immobilized fish trypsin at different PME concentrations, incubation times and temperatures (a) at 15°C; (b) at 25°C; (c) at 35°C.

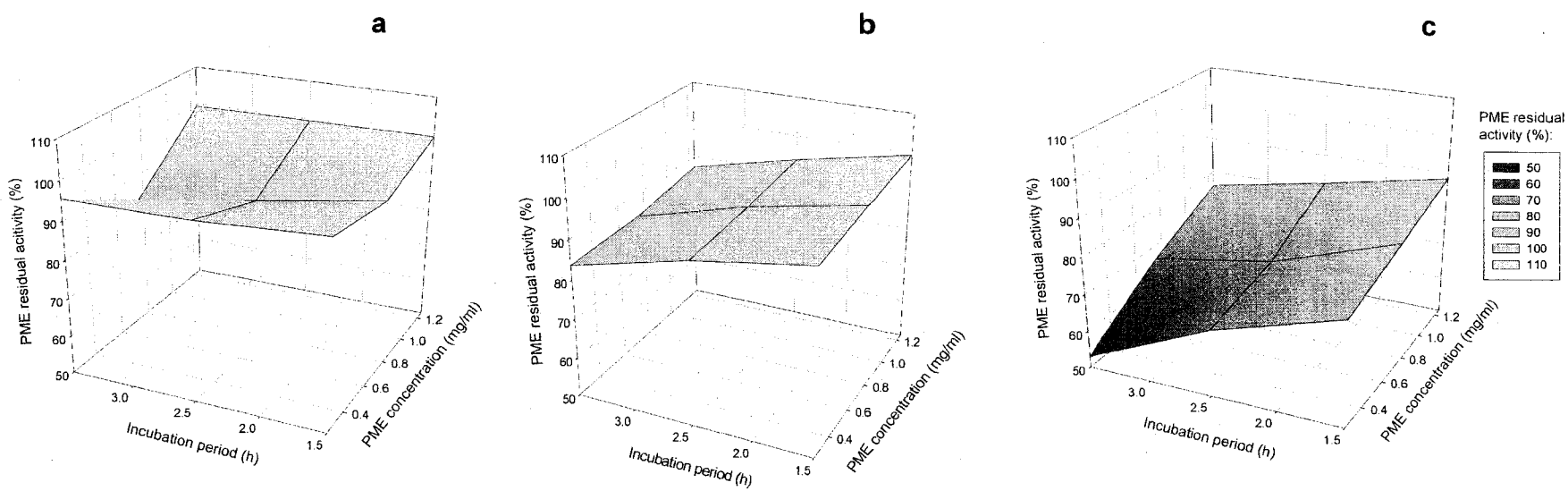


Fig. 6.2: Residual activity of PME treated by immobilized bovine trypsin at different PME concentrations, incubation times and temperatures (a) at 15ºC; (b) at 25ºC; (c) at 35ºC.

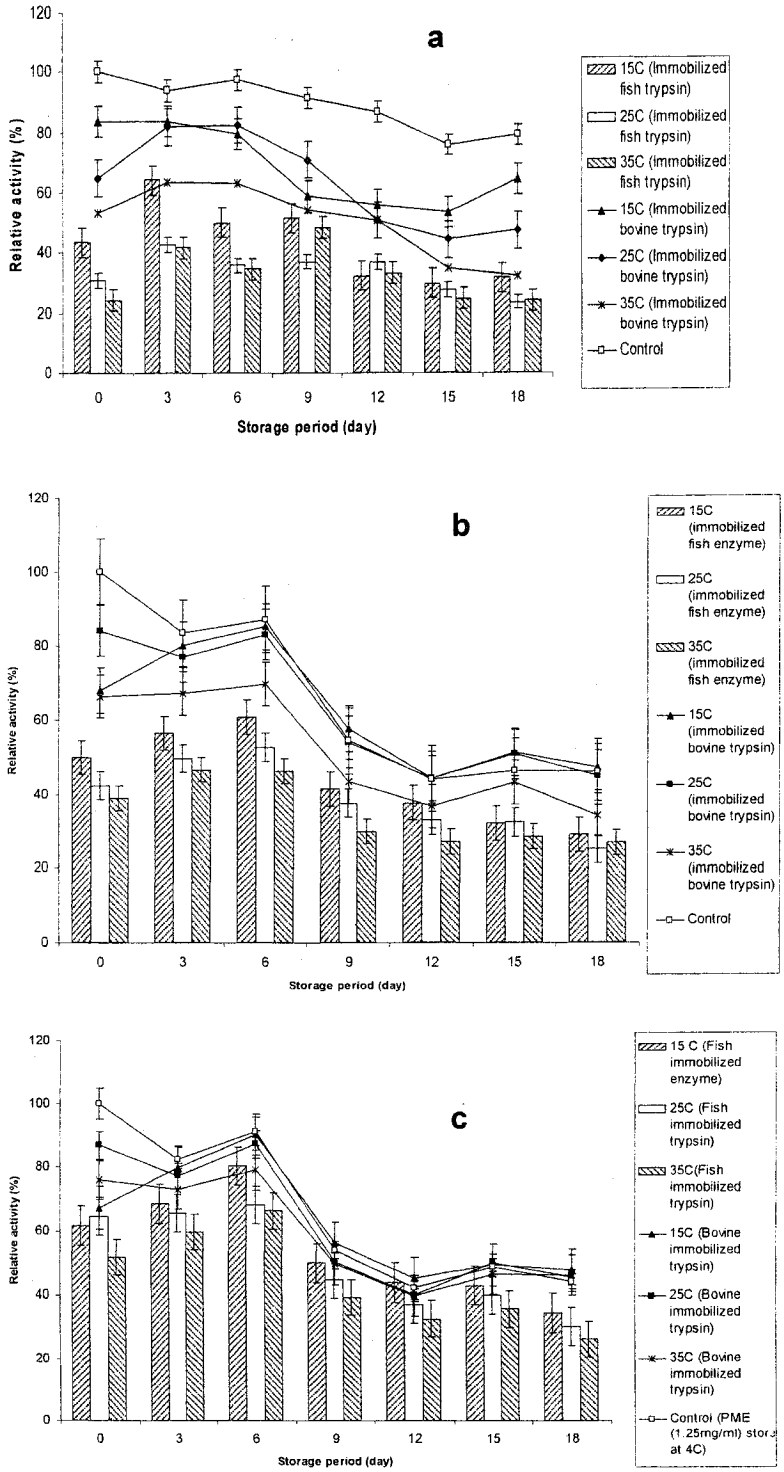


Fig. 6.3: Reactivation studies of immobilized bovine / fish trypsin treated PME with different initial PME concentration (a) 0.25 mg/ml; (b) 0.75 mg/ml; (c) 1.25 mg/ml.

Note: Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

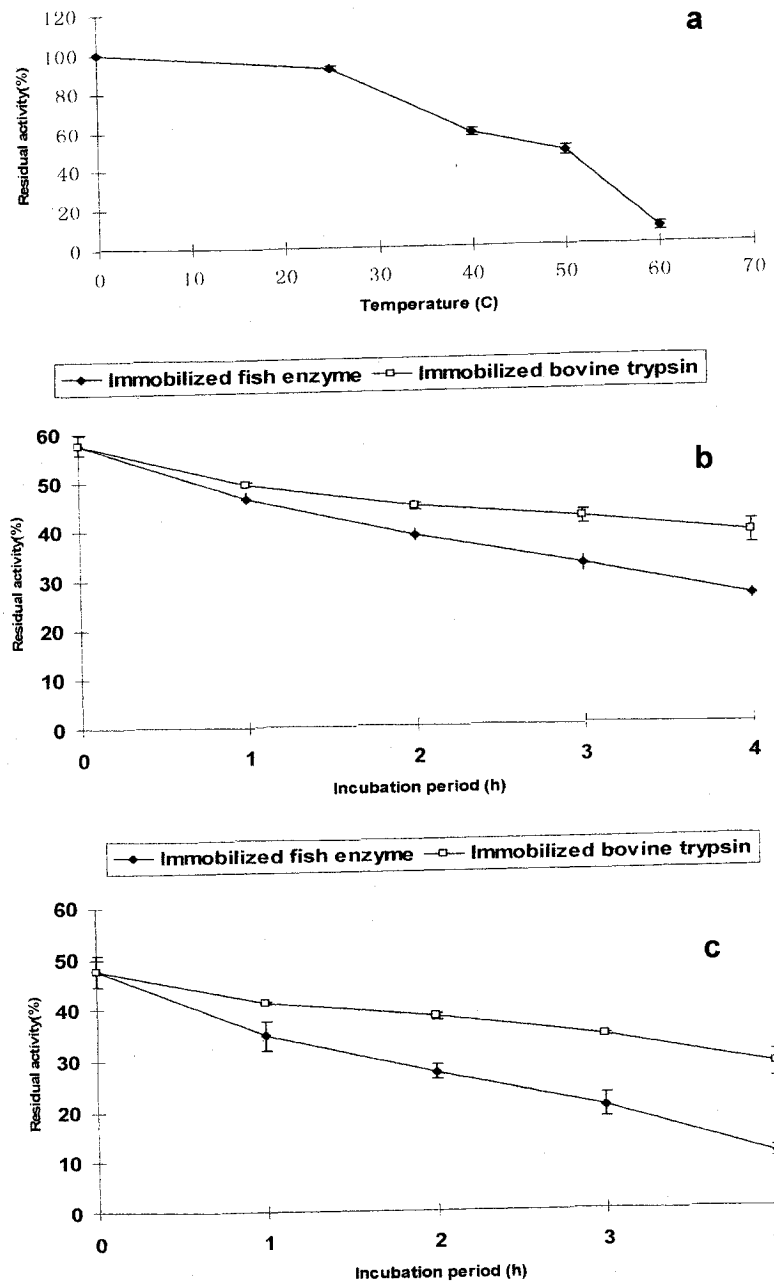


Fig. 6.4: Effect of heat treatment on PME activity (a) and residual activity of heated PME treated with immobilized fish / bovine trypsin after 30 min incubation at 40°C (b), and after 30 min incubation at 50°C (c).

Note: 1. The activity of PME at 0°C was as 100%.

2. Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

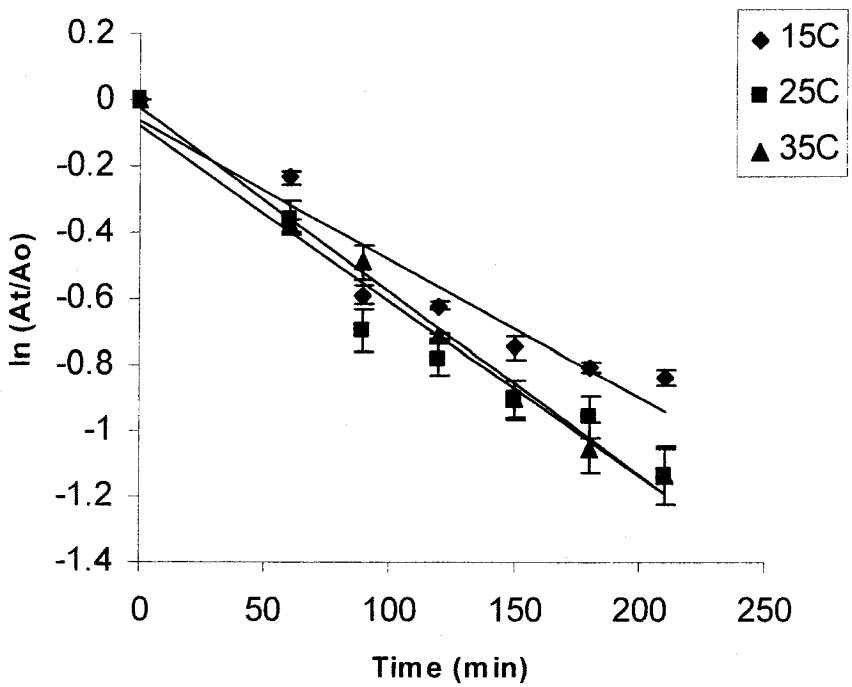


Fig. 6.5: Inactivation of PME with immobilized cunner fish trypsin at various incubation temperatures.

Note: Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

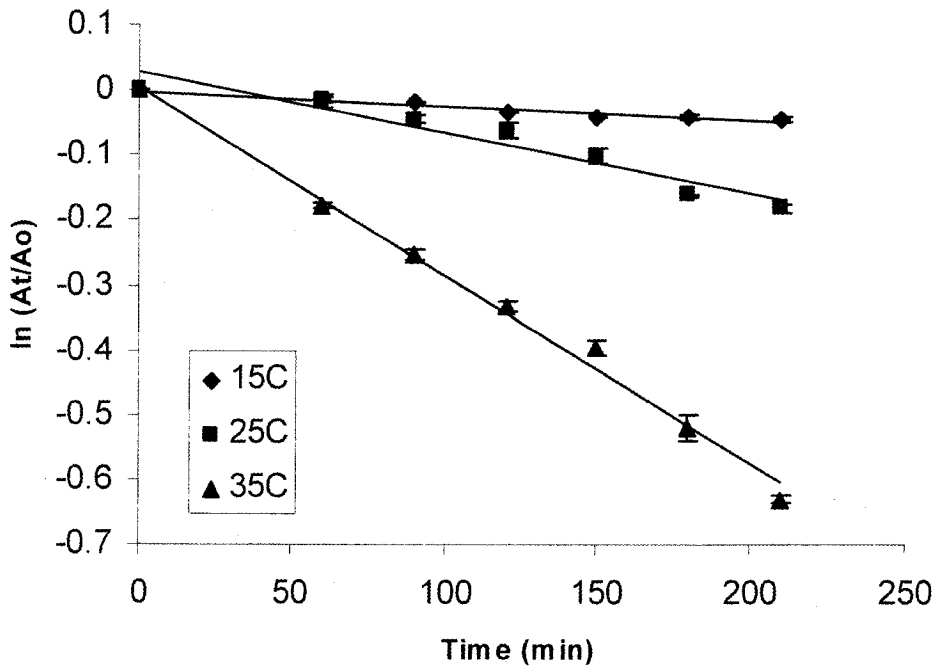


Fig. 6.6: Inactivation of PME with immobilized bovine trypsin at various incubation temperatures.

Note: Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

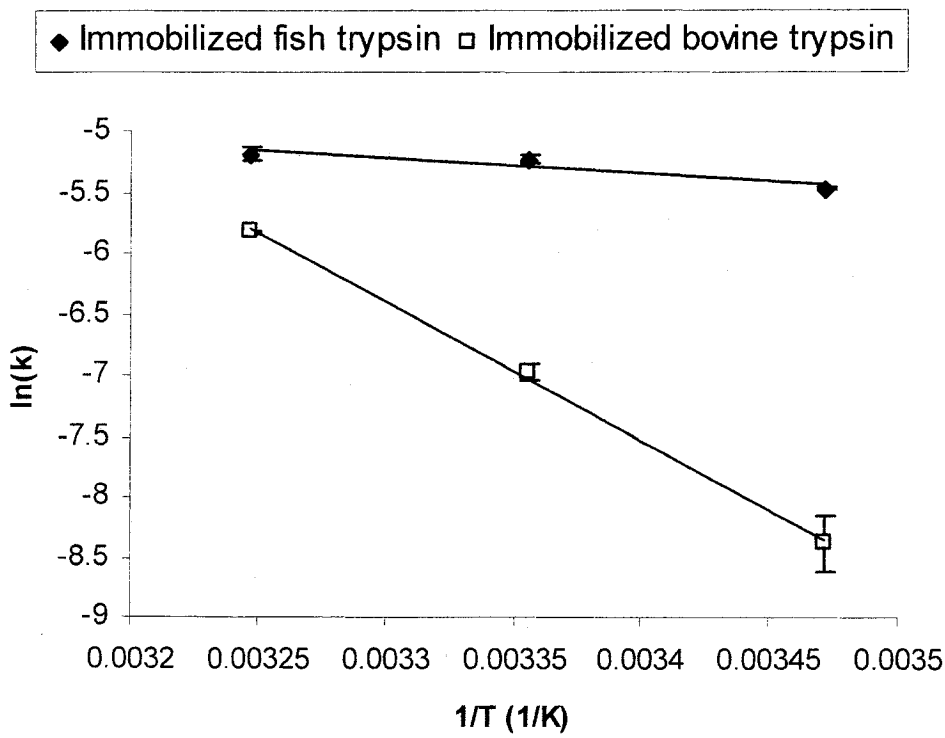


Fig. 6.7: Arrhenius plot of the rate constant of the inactivation of PME by immobilized cunner fish trypsin and immobilized bovine trypsin.

Note: Each point on the graph is the average of three replicates. The error bar shows the standard deviation among the three replicates.

CHAPTER VII

GENERAL CONCLUSIONS

1. Bovine trypsin was immobilized onto controlled pore glass (CPG) efficiently; about 60% enzyme protein incubated with CPG was bound onto the support material. Increasing the concentration of enzyme solution resulted in an increase in the amount of enzyme protein bound to the support material. The Langmuir theory could not explain the binding between the enzyme molecules and support material well due to the existence of multilayer binding of the enzyme molecules. The Freundlich empirical model fitted the practical conditions better than the Langmuir theoretical model. Because of the chemical binding between the protein molecules and support materials, the data fitted the Temkin model well.

2. The optimum conditions for immobilization were obtained with an initial trypsin concentration of 4 mg/ml, incubation time of 21 h, and a pH of 9.0 at reaction temperature 4°C. For the pH range investigated (pH 3.0 to pH 11.0), the optimum pH for storage stability of the immobilized bovine trypsin shifted to a relatively higher pH value (pH 9) compared to the free form of bovine trypsin (pH 3 & 5). The immobilized bovine trypsin may be used to facilitate the recovery of carotenoproteins. After 11 re-uses, the total yield of the product from the same immobilized bovine trypsin was 4.3 times higher than a single use of the same amount of the free enzyme.

3. The immobilization technique changed the properties of immobilized enzyme compared with its free counterpart, which includes the pH and / or temperature optimum, thermal stability, sensitivity to inhibition and kinetic properties. The immobilized bovine trypsin had higher optimum pH and temperature, higher thermal stability and higher K_m', and reduced sensitivity to inhibition by SBTI. The higher optimum temperature, thermal stability, and reduced sensitivity to the inhibitor make it possible to apply the immobilized enzyme in extreme conditions.

4. The presence of Ca^{2+} ions could enhance the amount of protein loading onto the support material, stabilize the immobilized enzyme during storage, and speed up the enzymatic reaction for immobilized enzyme.

5. Cunner fish trypsin was immobilized onto CPG using glutaraldehyde as cross-linking reagent. Maximum binding of the fish trypsin as well as optimum pH for storage stability were obtained at pH 9. The immobilization also changed the properties of the immobilized fish trypsin versus its free counterpart. The optimum pH and temperature were shifted from pH 8.5 to pH 9, and from 45°C to 50°C compared to the free enzyme.

6. Comparison of catalytic efficiencies (V_{max} / K_m') for both amidase and esterase reactions of the immobilized bovine and fish trypsins showed that the immobilized fish trypsin had higher catalytic efficiency value than its bovine counterpart, which indicated that the immobilized fish trypsin had

higher amidase and esterase activity than that of the immobilized bovine trypsin. The immobilized fish trypsin was evaluated by extracting carotenoprotein from shrimp shell. The immobilized fish trypsin retained about 75% of its initial hydrolytic capacity after 11 re-uses, and the yield obtained was over 20% higher than that of immobilized bovine trypsin.

7. The immobilized fish trypsin was used as a tool to inactivate pectin methylesterase (PME). The inactivation of PME was effective and about 70% of PME activity was lost by the immobilized fish trypsin. Comparing with immobilized bovine trypsin, the immobilized fish trypsin was more efficient on both native PME and partially heat-denatured PME. However, the inactivated PME could regain activity during storage at 4°C.

8. The kinetic studies on PME inactivation by immobilized fish / bovine trypsin showed that the inactivation rate constants increased and *D*-values decreased with increasing temperature for both immobilized fish and bovine trypsin. The inactivation rate constants of immobilized fish trypsin at various temperatures were higher than those of immobilized bovine trypsin. The activation energy (E_a) of the PME inactivation process by immobilized fish trypsin was lower than that by immobilized bovine trypsin, which explains the high catalytic capacity of the immobilized fish trypsin at various temperatures.

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APPENDIX 1

Data presented in this appendix are statistical information referred to in chapter III

Table A1-1 ANOVA for amount of protein bound

Source of Variance	DF	Sum of Squares	Mean Square	F Value	P Value*
Incubation time	2	1.71	0.85	15.20	0.0013
Trypsin concentration	2	3.74	1.87	33.31	<0.0001
Incubation time * Trypsin concentration	4	1.30	0.32	5.81	0.0136

Table A1-2 ANOVA for activity of immobilized bovine trypsin

Source of Variance	DF	Sum of Squares	Mean Square	F Value	P Value*
Incubation time	2	2267.88	1133.94	424.91	<0.0001
Trypsin concentration	2	2110.47	1055.23	395.42	<0.0001
Incubation time * Trypsin concentration	4	4596.58	1149.15	430.61	<0.0001

* If the P values are < 0.01, the factors are statistically significant at 99% confidence level; if the P values are < 0.05, the factors are statistically significant at 95% confidence level.

APPENDIX 2

Data presented in this appendix are statistical information referred to in chapter VI

Table A2-1 ANOVA for PME inactivation by immobilized fish and bovine trypsin

Source of Variance	DF	Sum of Squares	Mean Square	F Value	P Value*
Immobilized enzyme	1	19956.11	19956.11	3063.44	<0.0001
PME concentration	2	10209.06	5104.53	783.59	<0.0001
Incubation time	2	5230.05	2615.03	401.43	<0.0001
Incubation temperature	2	6307.12	3153.56	484.10	<0.0001
Incubation time * PME concentration	4	280.62	70.15	10.77	<0.0001
Incubation temperature * PME concentration	4	250.50	62.62	9.61	<0.0001
Incubation time * temperature	4	811.05	202.76	31.13	<0.0001
Incubation time * temperature * PME concentration	8	199.66	24.96	3.83	0.0005
Immobilized enzyme * PME concentration	2	6643.98	3321.99	509.95	<0.0001
Immobilized enzyme * incubation time	2	164.20	82.10	12.60	<0.0001
Immobilized enzyme * incubation temperature	2	1471.81	735.91	112.97	<0.0001
Immobilized enzyme * incubation time * PME concentraton	4	43.17	10.79	1.66	0.1654
Immobilized enzyme * incubation temperature * PME concentraton	4	1483.59	370.90	56.94	<0.0001
Immobilized enzyme * incubation time * temperature	4	47.38	11.85	1.82	0.1305
Immobilized enzyme * incubation time * temperature * PME concentraton	8	60.87	7.61	1.17	0.3251

* If the P values are < 0.01, the factors are statistically significant at 99% confidence level; if the P values are < 0.05, the factors are statistically significant at 95% confidence level.